University of Alberta

Purification and identification of two aluminum-binding exudate proteins from the aluminum-sensitive wheat cultivar Katepwa

by

Brenda Yei Chow

A thesis submitted to the Faculty of Graduate Studies and Research in partial fulfillment of the requirements for the degree of Master of Science

Department of Biological Sciences

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Date 15 September 1999

Quotes overheard in the Taylor Lab (1996-1999)*:

"No really, *this* flood was on purpose. The floor around the sink looked like it *needed* a good mopping …"

- with an Indonesian accent

"Is it lunch time yet???"

"Oh Mighty Lab Gnome, please take these test tubes as offerings for peace and success with my experiment…"

- chanted over and over

"supposebly"

"The fumes stung my eyes, so I called Greg and campus security!"

- with a Colombian accent

"Bite me."

"I eat raw bacon and wash it down with homo milk. Mmmmmm, good."

- with a Polish accent

"Is it lunch time yet???"

"a-LOOM-inium."

"No, *you* ask if we can borrow his centrifuge…"

* Names omitted to protect the misquoted.

University of Alberta

Faculty of Graduate Studies and Research

The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies and Research for acceptance, a thesis entitled *Purification and identification of two aluminum-binding exudate proteins from the aluminum-sensitive wheat cultivar Katepwa* submitted by Brenda Yei Chow in partial fulfillment of the requirements for the degree of Master of Science.

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Gregory J. Taylor (supervisor)

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Date 10 September 1999

ABSTRACT

Two exudate proteins were purified from the growth media of an Al-sensitive wheat cultivar (*Triticum aestivum* L. cv. Katepwa) in an effort to identify possible sites of Al-induced injury. While a suite of at least 14 polypeptides was exuded into the media under non-stress conditions, only two major polypeptides (24 and 40 kD) were detected from seedlings in high Al concentrations. These two proteins also bind Al strongly, as shown through immobilized metal ion affinity chromatography. Protein purification was achieved with a single procedure using continuous elution gel electrophoresis. Tryptic fragments from the 24 and 40 kD proteins were sequenced and are homologous with germin-oxalate oxidase and serine proteinase inhibitor (serpin) respectively. Oxalate oxidase activity was confirmed in exudates using an in-gel detection method. Although both proteins appear to originate in the seed, histochemical staining demonstrated that oxalate oxidase is also localized to mature root regions of non-stressed roots. In contrast, treatment with Al resulted in staining along the entire root length (tip included). Inhibition of oxalate oxidase activity could be shown using oxalate oxidase from barley roots and Katepwa exudates. These results suggest that apoplasmic/rhizospheric oxalate oxidase is a potential target of Al toxicity.

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LIST OF ABBREVIATIONS

Al.....aluminum

ALA.....aminolevulinic acid

ATPase.....adenosine 5'triphosphatase

bromophenol blue.....3', 3', 5', 5'-tetrabromophenolsulphonphthalein

BSA.....bovine serum albumin

^{o}C.....degrees Celsius

cDNA.....complementary DNA

cv.....cultivar

DNA.....deoxyribonucleic acid

DTT.....dithiothreitol

E. coli.....*Echerichia coli*

EDTA.....ethylenediaminetetraacetic acid

ER.....endoplasmic reticulum

FW.....fresh weight

x g.....multiplied by the standard acceleration of gravity

GDP.....guanosine 5'-diphosphate

HSP.....heat shock protein

IDA.....iminodiacetate

kD.....kiloDalton

l; $l_{t=0}$.....length; length at time zero

µg; g.....microgram; gram

µL; mL; L.....microliter; milliliter; liter

µM; mM; M.....micromolar; millimolar; molar

µmol.....micromole

mAmp..... milliAmpere

mm; m; cm.....millimeter; meter; centimeter

min.....minute

mN; N.....millinormal; normal

mRNA.....messenger ribonucleic acid

nm; mm; cm.....nanometer; millimeter; centimeter

PAGE.....polyacrylamide gel electrophoresis

RNA.....ribonucleic acid

rpm..... revolutions per minute

Rubisco.....ribulose 1,5-bisphosphate carboxylase-oxygenase

s.....second

SDS.....sodium dodecyl sulphate

Tris.....tris (hydroxymethyl) aminomethane

v/v; v/w; w/v.....volume per volume; volume per weight; weight per volume

1. INTRODUCTION

1.1. GENERAL INTRODUCTION

Aluminum (Al) is the most abundant metal in the earth's crust. It becomes biologically accessible through the natural processes of weathering and erosion. The overall chemistry of Al is complex because many forms can co-exist depending on soil pH (Kinraide 1991). In neutral and basic environments, Al exists predominately as a precipitate or in non-toxic forms (e.g. $Al(OH)_3$ and $Al(OH)_4^-$). As the solution becomes less basic (approaching pH 7), Al successively protonates to yield $Al(OH)_2^+$ and $Al(OH)^{2+}$. In acidic environments (pH < 5), the phytotoxic species Al^{3+} dominates. The threat of Al injury to agricultural crops in acid soils has been well established. It has been estimated that up to 40 % of the world's arable soils are predisposed to Al toxicity (Haug 1984), including the Peace River area of Alberta and much of eastern Canada. Worldwide, plants in the tropics and subtropics are especially prone to Al-induced injury because of a more naturally acidic soil. Aluminum injury can be exacerbated by many agricultural and industrial activities (e.g. nitrogen-fertilization and combustion of fossil fuels), even in sites not previously prone to Al toxicity. Thus, Al is an important factor limiting food production in Canada and many developing countries. Currently, intense research is underway to develop Al-resistant cultivars of important crop species (e.g. de la Fuente *et al.* 1997; MacDiarmid and Gardner 1998). For this to succeed, we must gain a thorough understanding of how Al affects growth.

1.2. ALUMINUM-INDUCED INJURIES ARE LOCALIZED AT THE ROOT TIP

Although the effects of Al are evident throughout the plant, roots are generally recognized as the primary site of injury. Characteristically, roots exposed to toxic levels

of Al are stubby and brittle, with thickened brown laterals and tips (Foy, Chaney and White 1978). Roots lack fine branching and become stunted overall. As a result of this damage, nutrient deficiency and drought symptoms appear secondarily. Other symptoms of Al toxicity are apparent at the cellular level. Cells of the root cap become vacuolated, plastid development and Golgi function are disrupted, and nuclear structure becomes altered (Taylor 1988). In addition, epidermal, endodermal and cortical cells of the root autolyze (Wagatsuma *et al.* 1995), and vascular elements become indistinguishable from root cap cells (Fleming and Foy 1968). Common physiological effects include: disruption of Ca homeostasis (Huang *et al.* 1992a; Huang *et al.* 1992b; Huang *et al.* 1994) and signal transduction (Haug *et al.* 1994); stimulation of callose deposition (Zhang *et al.* 1994); inhibition of ion transport processes (Rengel and Elliott 1991; Ryan *et al.* 1992); reduction in cell division and elongation (Horst 1995; Ryan *et al.* 1993); and disruption of plasma membrane structure and function (Suhayda and Haug 1986; Zhao *et al.* 1987). These toxic effects are localized at the root tip and many are observable within minutes or hours of exposure. Other evidence linking the root tip to Al injury includes the observation that Al accumulates mostly at the root apex (Wagatsuma *et al.* 1987; Rincon and Gonzales 1992; Delhaize *et al.* 1993; Archambault *et al.* 1997). Furthermore, root growth can be inhibited when Al is specifically applied to the root cap and meristem, but not when applied to maturer root regions (Ryan *et al.* 1993). Finally, callose synthesis is higher in cortical cells of the root apex when compared to more distal portions (Zhang *et al.* 1994), suggesting that the root tip is the primary site of Al-induced injury.

1.3. PLANTS HAVE DIFFERENTIAL TOLERANCE TO ALUMINUM

Plants exhibit a wide range of growth responses to Al. *Hydrangea* and members of the tea family (Theaceae) accumulate Al in the apoplasm and vacuole without suffering any apparent toxicity symptoms (Rengel 1996). Certain plants adapted to acidic soils (e.g. *Melastoma malabathricum* and *Ischaemum barbatum*) will actually become stunted in the absence of Al, possibly due to H^+ toxicity that is alleviated by Al (Osaki *et al.* 1997). In comparison, most crop plants are relatively sensitive to Al. Within this group, a range of tolerance nonetheless exists. Cultivar differences provide an appropriate experimental system for studying the complexities of Al tolerance and toxicity.

Tolerance to Al is thought to be achieved by two mechanisms: cellular exclusion from sensitive sites and tolerance of accumulated Al (Taylor 1988). An exclusion mechanism is one that immobilizes Al at the root-soil interface, thereby restricting its movement into Al-sensitive sites. Examples of this include the formation of a plant-induced pH barrier in the rhizosphere (Degenhardt *et al.* 1998), exudation of organic acids and phosphate to form less toxic Al-complexes (Pettersson and Strid 1989; Basu *et al.* 1994; Ryan *et al.* 1995; Pellet *et al.* 1996), and immobilization by mucilage (Horst *et al.* 1982). Even though the apoplasm restricts the movement of most Al, small quantities have been measured in the cytoplasm (Lazoff *et al.* 1994; Rengel and Reid 1997; Taylor *et al.* 1999). These plants may still express resistance by employing mechanisms to internally detoxify Al. Ma *et al.* (1997) detected cellular chelation of Al by citrate, while Pfeffer *et al.* (1986) studied the sequestration of Al within the vacuole. Both of these mechanisms are thought to improve tolerance to Al.

Research is currently underway to develop crop plants with superior Al tolerance. One method is based on the ability of citrate to bind Al into a non-toxic complex. By over-expressing the bacterial citrate synthase gene, de la Fuente *et al.* (1997) increased the amount of citrate produced by root tissues of tobacco (*Nicotiana tabacum*) and papaya (*Carica papaya*). They concluded that the increased levels of synthesis and excretion were responsible for the decreased Al accumulation and improved root growth in transgenic plants exposed to Al. MacDiarmid and Gardner (1998) also used molecular techniques to improve Al tolerance by over-expressing a putative Mg^{2+} transporter in Al-sensitive yeast. Aluminum tolerance was presumably a consequence of enhanced Mg^{2+} uptake, which compensated for an Al-induced Mg^{2+} deficiency. Ezaki *et al.* (1999) also conferred tolerance to Al-sensitive yeast. By over-expressing a gene for a copper binding protein and a gene encoding a GDP-dissociation inhibitor in different cell lines, Al tolerance was acquired. The copper-binding protein was thought to restrict Al uptake in one cell line, while the GDP-dissociation inhibitor was thought to promote the release of Al from cells after uptake. Whether equivalent gene transformations can impart tolerance in plants is uncertain. Delhaize *et al.* (1999) transformed *Arabidopsis* with a gene encoding phosphatidylserine synthase in the hopes of maintaining membrane fluidity in the face of Al stress. Although expression of this gene in *Arabidopsis* produced the desired change in phospholipid composition, its effects on Al tolerance have yet to be confirmed. Preliminary data using tobacco suggested that over-expression of the phosphatidylserine synthase gene would not enhance tolerance in plants. Although these studies represent pioneering efforts towards the development of resistant germplasm, it is

unlikely that single gene manipulations can provide the degree of tolerance required under field conditions.

As already described, the expression of Al toxicity in plants is complex. Thus it is not surprising that the published literature abounds with a wide range of primary Al-induced injuries (e.g. Rengel *et al.* 1995; Horst *et al.* 1995). The identification and study of primary lesions in plants will not only allow us to understand how Al alters physiological and cellular processes, but may also initiate new studies involved in generating Al-tolerant germplasm.

1.4. ALUMINUM BINDS TO BIOLOGICALLY IMPORTANT MOLECULES

The prerequisite for a primary toxic lesion is binding with Al. Because of the ionic properties of Al, it can form a complex with a number of ligands in biological systems. Since Al has been found to affect many neurological and skeletal disorders in humans (Martin 1986), many studies investigating Al-binding have been performed in animal systems. Although relatively few studies have shown binding in plants, information from animal systems is relevant to plants since biological molecules are structurally similar across organisms.

Charged species of Al interact strongly via electrostatic binding with oxygen-donating moieties. This includes carboxylate, phosphate, and sulfate groups (Kochian 1995; Martin 1986). Consequently, Al can complex with organic acids, inorganic phosphate, polyphosphates (such as nucleotides), polypeptides, and proteins. Due to the large number of oxygen groups in phosphate compounds, they bind with Al relatively

strongly. It has been established that Al can form a complex with inorganic phosphate. An Al-phosphate precipitate has been identified on the root surfaces of plants (Clarkson 1967; Rasmussen 1968; McCormick and Borden 1972). This is expected to limit the entry of phosphate, thereby inducing phosphorous deficiency symptoms. Aluminum has also been localized to the phosphate groups of DNA. Through fractionation studies, Matsumoto *et al.* (1976) demonstrated an associated between Al and DNA in the nuclei of actively dividing cells. In forming a complex with DNA, Al may destabilize the double helix (Matsumoto *et al.* 1977; Karlik *et al.* 1980). This can result in a blockage of cell division and produce the root stunting commonly associated with Al injury (Clarkson 1969; Matsumoto 1991).

Polypeptides and proteins represent other possible targets of Al. Aluminum can bind with the reactive side groups of amino acids. For example, the negatively-charged side chains of glutamate and aspartate can bind to Al via carboxylate groups (Mercero *et al.* 1998). Adjoining methyl groups in the side chain further stabilize this bond. Similarly, binding has been shown *in vitro* with poly (L-aspartic acid) and poly (L-glutamic acid) (Putterill and Gardner 1988). Proteins also contain reactive sites accessible to Al. The strength of such bonds depends on the site of interaction. Possible sites include the aforementioned side groups. In addition to glutamate and aspartate, protein segments rich in serine and threonine have also been proposed as potential Al-binding sites. Binding could be achieved at their hydroxyl groups (Carter *et al.* 1989). Aluminum can also bind weakly to the sulfhydryl (SH)-group of cysteine (Macdonald and Martin 1988). Sulfhydryl groups have been identified as essential for the catalytic

activity and structural integrity in many enzymes (Vangronsveld and Clijsters 1994). Metal binding to SH-groups is thought to be responsible for the inhibition of such enzymes as nitrate reductase, malate dehydrogenase, (Mathys 1975), Rubisco (Stiborova *et al.* 1986) and δ-ALA-dehydratase (a chloroplastic enzyme involved in chlorophyll synthesis; Prassad and Prassad 1987). The result of such Al binding may be an alteration in protein conformation. When Al binds to the carboxylate-rich regions of Ca-calmodulin, its helical structure partially unfolds (Siegel and Haug 1983). Consequently, calmodulin loses its regulatory activity over other enzymes like phosphodiesterase (Macdonald and Martin 1988). Similarly, Al negatively affects the conformation of acid and alkaline phosphatases (Macdonald and Martin 1988).

Another mechanism by which Al can directly affect the functioning of proteins is through cofactor substitution. Aluminum can physically substitute for Mg^{2+} and Fe^{3+} as cofactors because it has a similar ionic radius (Macdonald and Martin 1988). Size is thought to have a more significant effect on binding properties than charge (Martin 1986). Substitution-based inhibition has been documented for a number of enzymes including 3',5'-cyclic nucleotide phosphodiesterase and ferroxidase (Macdonald and Martin 1988).

In addition to the primary effects caused by direct binding of Al, Al can also induce secondary enzyme effects. One way this can occur is by Al-induced substrate limitation. An example of this is Al complexation with catechol, the substrate of catechol-O-methyl transferase (Macdonald and Martin 1988). Another secondary effect

on enzymes is through the inhibition of regulatory enzymes. For example, Al-binding to calmodulin results in a change in the regulation of enzymes like cyclic nucleotide phosphodisterase, membrane-bound ATPase (Rengel 1996) and NAD^+ kinase (Slaski 1995).

1.5. APOPLASMIC INJURIES OF ROOTS GROWN IN ALUMINUM

Despite much research, the primary lesions of Al remain controversial. Although cytoplasmic lesions have been cited, attention has begun to focus on the root apoplam as an important site since it is the first cellular structure to experience Al stress. Some authors have suggested that an apoplasmic lesion may be capable of initiating most, if not all, cytoplasmic lesions observed upon Al exposure (Blamey *et al.* 1993; Rengel 1995). Since the Al toxicity problem is complex, it may be naïve to expect that a single lesion can be responsible for the suite of effects observed. Consequently, sites such as the cell wall and plasma membrane have been proposed as primary site of injury.

Initial uptake and accumulation of Al occurs in the cell wall (Zhang and Taylor 1990, Zhang and Taylor 1991), perhaps accounting for 30 to 99.5 % of total cellular Al (Tice *et al.* 1992; Archambault *et al.* 1996; Taylor *et al.* 1999). The cell wall consists of a cellulosic/hemicellulosic framework cemented together by a pectin matrix. Embedded within this carbohydrate matrix are enzymes and structural proteins. Pectin is a negatively charged polycarboxylate, with free carboxylate groups available for binding. These carboxylate groups can cross-link with Al to reduce cell wall elasticity and perhaps permeability (Klimashevskii and Dedov 1975). Blamey *et al.* (1993) showed that the solution flow through a synthetic cell wall made of calcium pectate was reduced by >80%

within 2 minutes after Al addition. This suggests that Al can rapidly target the cell wall, possibly accounting for the reduction in water movement into root cells and subsequent long-term drought and nutrient deficiency symptoms associated with Al toxicity. By binding to the cell wall, Al could also reduce cell elongation by increasing cell wall rigidity (Blamey *et al.* 1993). Aluminum has also been shown to inhibit magnesium uptake via competitive binding at exchange sites in the root cortical cell wall (Keltjens 1995). In addition to the cell wall, Al appears to target the plasma membrane. Aluminum-induced injuries at the plasma membrane include: an increased permeability to nonelectrolytes (Zhao *et al.* 1987); an increase in potassium leakage from the cytosol (Wagatsuma *et al.* 1995); and a blockage of inward-directed calcium channels (Rengel and Elliott 1991; Rengel 1992).

1.6. COMPONENTS OF THE RHIZOSPHERE

The rhizosphere may represent another potential site of Al injury. By definition, the rhizosphere is the narrow region of soil immediate to, and subject to the influences of, living roots (Curl and Truelove 1986). Although the rhizosphere was first identified as the bacterial-rich region surrounding legume roots, it is now realized that all plants release compounds into the rhizosphere throughout growth and development, regardless of bacterial association (Curl and Truelove 1986). Using radioactive labeling, McDougall and Rovira (1970) observed that more compounds are exuded from the root tip than more mature regions. The total amount of carbon released has been estimated to be 3 to 13 % of net photosynthetically-fixed carbon. This value is dependent on plant species, development stage, environmental stress, and collection conditions (Bekku *et al.* 1997). The process by which these compounds are released into the rhizosphere is

termed exudation. Spatially, the rhizosphere is a continuum of space, where exudates may either be closely associated with the cell wall (tightly or loosely bound) or "free" within the rhizosphere. The region closer to the root surface is more likely to contain molecules from root cells; regions further away from the root surface are less likely to contain exudates from those cells, but more likely to contain molecules from neighboring plants or organisms. Although the rhizosphere technically defines a region of soil, many studies have used the bulk solution as a representative system. This collection method tends to identify loosely cell wall-associated and "free" proteins (tightly wall-associated proteins are omitted), but offers the distinct advantage of minimizing root damage during removal from the growth media.

Exudates consist of a multitude of macromolecules, many of which are unique to the rhizosphere (Costa *et al.* 1997). Exudates can be categorized into low and high-molecular weight compounds. Low molecular weight compounds consist mostly of amino acids, phenolics, organic acids, and sugars. Many of these have well-established roles in the rhizosphere. For example, phytosiderophores are exuded from cereal species like oats and barley (Zhang *et al.* 1991; Brown *et al.* 1991). They are composed of amino acids that act as chelators for iron acquisition from soils. In contrast, allelochemicals are exuded to suppress the growth of competing plants. These consist of a range of molecules including phenolics from chickweed (Inderjit and Dakshini 1998), sorgoleone from *Sorghum* (Einhellig *et al.* 1993) and cinnamic acid from cucumber (Yu and Matsui 1997). Another class of exudates consists of signaling compounds used for establishing symbiotic relationships. This includes flavonones from *Vicia sativa* (Zaat *et al.* 1989),

flavonoids from *Trifolium repens* (Janczarek *et al.* 1997), and chalcone from *Robinia pseudoacacia* (Scheidemann and Wetzel 1997). A number of plants also exude organic acids that are thought to be involved with iron acquisition, phosphorus acquisition, and Al detoxification (Basu *et al.* 1994b; Ryan *et al.* 1995; Pellet *et al.* 1996). Although amino acids and sugars have also been detected in root exudates (Vancura 1964; Vancura and Hovadik 1965), it has been shown that they are subsequently re-acquired by roots and microorganisms to support growth (Darrah 1996).

The high molecular weight component of exudates consists of mucilage and a variety of enzymes. Mucilage is a high-molecular weight material composed of polysaccharides (Marchner 1997). Root surfaces are coated with this material, especially in the apical zone. Secretion of mucilage occurs from both root cap and epidermal cells (Marschner 1997). The primary function of mucilage is in protecting the root from desiccation and mechanical damage during growth through the soil. A wide spectrum of biologically important enzymes is also released into the rhizosphere. Many of these enzymes are involved with nutrient acquisition. Acid phosphatase, an enzyme which hydrolyzes organic phosphorus into plant-available orthophosphates, has been identified in sterile exudates of gram (*Cicer arientinum*), clover (*Trifolium alexandrinum*), wheat (*Triticum aestivum*) and mustard (*Brassica nigra*) (Chhonkar and Tarafdar 1981). Acid phosphatase activity in lupin exudates is stimulated under conditions of phosphate deficiency, suggesting that secretion is also an adaptive mechanism for growth in phosphorus deficient soils (Li *et al.* 1997). Invertase, ribonuclease and deoxyribonuclease activities have been identified in sterile root exudates from corn

(Chong and Bandurski 1963). It is believed that each of these enzymes solubilize macromolecules into smaller units, allowing for easier uptake and utilization by the root. Peroxidase is another enzyme identified in the root exudates of creeping fescue, alfalfa, mustard and cress (Vaughan *et al.* 1994; Gramss and Rudeschko 1998). Peroxidase has been recognized as a mediator of cell wall properties. It cross-links portions of the cell wall, thereby reducing extensibility. Peroxidase also assembles lignin and protein components of the cell wall (Lee and Lin 1995). Enzyme activities of laccase (involved in lignin degradation), aromatic-ring cleavage dioxygenase (involved in meta-cleavage pathway), and ascorbate dioxygenase (involved in the conversion of ascorbate and proline into hydroxyproline,which is used in the synthesis of fibrous proteins in the extracelluar matrix) have also been detected in the exudates of various crop plants (Lane 1994; Gramss and Rudeschko 1998). Superoxide dismutase was also recently detected in the exudates of a crop plant (U Basu, personal communication).

1.7. PROTEIN SECRETION TO THE APOPLASM/RHIZOSPHERE

Proteins in the rhizosphere arrive via secretion into the cell wall space (Curl and Truelove 1986). Secretion is defined as the process of flow through the endomembrane system to the cell exterior (Jones and Robinson 1989; Chrispeels 1991; Satiat-Jeunemaitre *et al.* 1996). Unlike proteins targeted to intracellular compartments, this flow of proteins (also termed the default pathway) describes the route that lacks specific protein receptors. This pathway continuously secretes proteins by bulk flow; newly synthesized proteins may exit the cell after only 20 minutes (Jones and Robinson 1989).

In an overview, secretion involves synthesis and modification in the endoplasmic reticulum (ER), processing in the golgi apparatus, movement through the cell via transport vesicles, vesicle fusion with the plasma membrane, and finally export to the cell surface (Jones and Robinson 1989; Chrispeels 1991; Satiat-Jeunemaitre *et al.* 1996). This process initiates with protein synthesis on ribosomes in the cytosol. Translation of a 13-30 amino acid sequence targets the nascent polypeptide into the ER. This fragment is recognized by a signal recognition particle on the ER surface, a prerequisite for entry into the secretory pathway. The signal peptide is cleaved co-translationally, thereby releasing the protein into the ER. Many proteins may also be co-translationally glycosylated within lumen of the ER. Proteins are folded, processed (e.g. glycosylated), and assembled into oligomers as specified. Proteins that are not properly assembled will form aggregates and are subsequently broken down. Intact proteins enter transport vesicles from the ER and fuse to the cis surfaces of the golgi. Here, proteins are further processed to yield mature glycoproteins. Secretory vesicles bud off from trans golgi surfaces to deliver the mature protein to the plasma membrane. How these vesicles recognize the plasma membrane has not yet been established. Vesicles are thought to recognize the plasma membrane by the "fusion machinery" consisting of membranous and cytosolic proteins. Recognition may also be regulated by intracellular calcium concentration. Upon fusion with the plasma membrane, proteins are then excreted into the cell wall space. Depending on their function, proteins may either remain mobile or become fixed into position within the cell wall (Varner and Lin 1989). Charge associations largely determine their position within this space in proximity to the cell wall. Proteins released into the rhizosphere may originate via passive diffusion from this cell wall space.

1.8. LIMITATIONS OF EXUDATE STUDIES

Many studies examining exudates have been flawed by the nature of their design. The most obvious source of error in many studies occurs when aseptic conditions are not maintained throughout the experiments. In such cases, it is difficult to determine the origin of putative exudate molecules since both bacteria and fungi are known to exude compounds into the environment (Curl and Truelove 1986). For example, Praveen-Kumar and Burman (1996) assayed a large range of plants and concluded that 13 species exude nitrate reductase. No special precautions, however, were taken to ensure sterility. Consequently, their conclusion of a root origin may be invalid since nitrate reductase is also a bacterial enzyme (Antipov *et al.* 1998). Numerous studies have also claimed a root origin for phosphatase and peroxidase. Even though both have been identified as *bona fide* exudate proteins of certain plants (e.g. Vaughan *et al.* 1994; Li *et al.* 1997; Gramss and Rudeschko 1998), other studies cannot rule out the possibility of microbial contamination (e.g. Tadano *et al.* 1993; Lee and Lin 1995; Dinkelaker and Marschner 1992). Another possible source of error comes from injured or dying cells. As the root grows through a solid medium like soil, cells at the root tip become abraded and sloughed off. This becomes a concern as cells are mechanically disrupted to release their cytoplasmic contents. Barber and Gunn (1974) observed that barley roots grown in glass ballotini (uniform glass beads) exude nearly twice the amount of amino acids and carbohydrates as those grown in solution. This enhanced exudation could actually reflect physical damage to the cell. Hence, a cytoplasmic origin cannot be dismissed when a solid medium is employed. Because these two sources of contamination are possible, the methods used in obtaining plant exudates must be clearly defined before the term "exudate" can be legitimately applied.

1.9. WORKING HYPOTHESIS

Aluminum is the major factor limiting plant growth in most acidic soils. The expression of Al injury is complex, and several sites of injury have been proposed. In plants, primary lesions have been localized in both the cell wall and plasma membrane. Since biologically important enzymes have been identified in the rhizosphere, they could also represent sites of Al-induced injury. In an attempt to identify potential sites of Al injury, the goals of my research were to 1) purify Al-binding proteins from the growth medium supporting the growth of an Al-sensitive cultivar of wheat, 2) identify the isolated proteins, and 3) if possible, characterize the effect of Al on the activity of the purified enzymes. To date, no published studies have focused on the toxic effects of Al on exudate proteins. The information gained from my study may initiate further research into developing Al-resistant germplasm.

2. MATERIALS AND METHODS

2.1. GROWTH OF ALUMINUM-SENSITIVE *TRITICUM AESTIVUM* CULTIVAR KATEPWA

Triticum aestivum cv. Katepwa is currently the most widely grown red spring wheat in Canada. Since it is relatively sensitive to Al and its root exudate profile has been previously studied (Basu *et al.* 1994a), Katepwa was chosen for the present experiments.

Seeds were surface sterilized in 10 % bleach for 20 minutes under constant agitation and rinsed for approximately one minute with Milli-Q filtered water (Millipore). Seeds were germinated overnight in an aerated solution of 0.005 $g \cdot L^{-1}$ of Vitavax (fungicide). Germinated seeds were plated onto mesh suspended over 10 L of nutrient solution containing (in µM): 3300 NO_3^-,1000 Ca, 800 K, 300 Mg, 300 NH_4^+, 100.65 SO_4^{2-}, 100 PO_4^{3-}, 34 Cl, 20.2 Na, 10 EDTA, 10 Fe, 6 B, 2 Mn, 0.5 Zn, 0.15 Cu, and 0.1 Mo (pH 4.3). This solution was made using the following salts: $Ca(NO_3)_2 \cdot 4H_20$, $Mg(NO_3)_2 \cdot 6H_2O$, NH_4NO_3, K_2HPO_4, K_2SO_4, KNO_3, $MnCl_2 \cdot 4H_2O$, H_3BO_3, $ZnSO_4 \cdot 7H_2O$, $CuSO_4 \cdot 5H_2O$, $Na_2MnO_4 \cdot 2H_2O$, $FeCl_3 \cdot 6H_2O$, and Na_2EDTA. Seedlings were grown initially for two days in the dark (to allow for root establishment), and then left for an additional day under light conditions. Healthy seedlings of approximately the same size were transferred onto a nylon mesh suspended on rafts in 500 mL beakers (five seedlings per beaker). Beakers were filled with nutrient solution (400 mL) containing 0, 50, 100, 150 or 200 µM $AlCl_3$ (pH 4.3; three beakers per Al treatment). Plants were grown in a controlled environment chamber with 16 hours light (24°C, 68 % relative humidity) and 8 hours darkness (20°C, 85 % relative humidity) under constant aeration. The

photosynthetic photon flux was 335 ± 12 µmol $m^{-2}s^{-1}$ at plant base level. A mixture of fluorescent and incandescent lights was used in the chamber. Root length of three plants from each beaker was measured every 24 hours over a 72 hour period to give an average root length. Measurements were taken against plastic-coated graph paper.

2.2. DETERMINATION OF CALLOSE

After 72 hours of growth, three root tips (10 mm) per plant were harvested for callose determination (three plants harvested per beaker, three replicate beakers per treatment). Following excision, root tips were stored overnight at 4°C in tubes containing 1.4 mL 95 % ethanol. Callose quantification was conducted using the spectrofluorometric technique described by Zhang *et al.* (1994). The ethanol from each tube (containing three root tips) was decanted and replaced with 0.2 mL 1N NaOH. Samples were then homogenized for 20 seconds using a micro-pestle mounted on an electric drill. The pestle was rinsed with 0.8 mL of 1.0 N NaOH after each grinding to a final volume of 1.0 mL per tube. The tubes were heated in an 80°C water bath for 15 minutes and then centrifuged for three minutes at 15000 x g. The developing mixture for the assay contained: 0.3 mL 1 N NaOH, 0.8 mL 0.1 % aniline blue, 0.42 mL 1 N HCl, and 1.18 mL 1 M glycine buffer (adjusted to pH 9.5 using solid NaOH). To the developing mixture, 100 µl supernatant was added. Following two incubation periods (50°C for 20 minutes and 23°C for 30 minutes), callose was quantified using a spectrofluorometer (AMINCO-Bowman, Series 2) with an excitation wavelength of 398 nm and an emission wavelength of 495 nm. A standard curve was made using Pachyman

(polyglucose; Calbiochem) for comparison. Callose values were expressed as Pachyman equivalents per gram fresh root weight (g Pachyman equivalent·g^{-1} FW).

2.3. COLLECTION OF GROWTH MEDIA

The bulk solution bathing roots during growth was used to represent the rhizosphere. It is recognized that this method of protein collection provides information on only a portion of the rhizosphere components, namely the "free" and loosely bound rhizosphere proteins. Since such proteins are expected to be easily accessible to environmental Al, this method is valid for the aims of this project. To ensure that the proteins collected were of plant origin (not bacterial or fungal), aseptic techniques were used throughout all experiments. All procedures that involved opening sterile containers were conducted in a Laminar flow hood. Seeds were surface sterilized in 0.01 % $HgCl_2$ for 10 minutes under constant agitation, and then imbibed in sterile Milli-Q filtered water for 6 hours. Seeds were then transferred onto petri dishes containing a solidified germination medium composed of ($g{\cdot}L^{-1}$): 8.0 agar, 0.108 myosin, 2.0 casein, and 34.23 sucrose. Growth chamber conditions were as described above. After an initial 3 day growth period, sterile seedlings were transferred onto a nylon mesh suspended on polypropylene rafts over sterile nutrient solution (as described previously). Magenta vessels (Sigma) were used to enclose the sterile seedlings, with 5 plants per 80 mL solution. During the 7 to 10 day growth periods, vessels containing seedlings and growth medium were placed on a shaker (60 rpm) inside the growth chamber. To maintain a low a pH (approximately pH 4.3), 120 µl of 200 mM NH_4Cl was added to the vessels every three days.

To collect the growth medium, seedlings were gently removed to limit root damage. The medium in each vessel was tested for sterility by plating aliquots (120 µl) onto sterile petri dishes containing solidified germination medium (as described above). The remainder of the growth solution was stored at –20 °C until further processed. Before inspection for bacterial and fungal growth, aliquots on the solidified media were incubated for at least 3 days under the same growth chamber conditions used for growing the plants. Previous incubations for up to two weeks showed that a 3 day period was sufficient time for colonies to grow if contamination was present. Following confirmation of sterility, solutions from each vessel were pooled and concentrated.

Sterile solutions were concentrated at 4°C with a pressure ultrafiltration system (Amicon 8200) using a YM10 membrane (10 kD exclusion limit; Amicon). The low molecular weight filtrate was discarded while the high molecular mass component was concentrated further under vacuum using a Speed Vac concentrator (Savant SC100). Proteins were then syringe filtered (cut off of 10 kD) and stored at –20°C in concentrations ranging from 0.3 to 0.8 $\mu g \cdot \mu L^{-1}$.

To collect exudates following Al exposure, seedlings were grown similarly to the aseptic method described above. After three days in petri dishes, sterile seedlings were transferred to Magenta vessels containing sterile nutrient solution. Roots were allowed to establish for 2 days in this solution before being transferred into Al solutions. Plants were exposed to 0, 100, 150 and 200 µM $AlCl_3$ in sterile nutrient solution (pH 4.3) for 5 days, and the solutions were then tested for sterility.

2.4. QUANTIFICATION AND VISUALIZATION OF PROTEINS

Proteins in the growth medium were quantified using the Bradford microassay (Bradford 1976). Bovine serum albumin was used as a standard, and absorbance was measured at 545 nm. Protein profiles were visualized using SDS-PAGE following the method of Laemmli (1970). Samples of protein (3 - 10 µg) were mixed with an equal volume of sample buffer (0.125 M Tris-Cl pH 6.8, 4 % [w/v] SDS, 20 % [v/v] glycerol, 10 % [v/v] 2-mercaptoethanol) and boiled for 3 minutes prior to loading. Protein profiles were analyzed using the Miniprotein II system (Bio-Rad). Gel dimensions were 8 cm width x 7.3 cm length, with a thickness of 1.0 mm. Samples were electrophoresed at 10 mA through the stacking gel (4 % total monomer concentration, 2.7 % cross-linking monomer solution; pH 6.8) and 20 mA through the separating gel (12.5 % total monomer concentration, 2.7 % cross-linking monomer solution; pH 8.8). The tank buffer consisted of 0.025 M Tris (pH 8.8), 0.192 M glycine and 0.1 % (v/w) SDS.

Proteins were stained with silver nitrate following a modified method of Morrissey (1981). After electrophoresis, proteins were immobilized in 45 % (v/v) methanol and 12 % (v/v) acetic acid overnight. The gel was then transferred into 10 % (v/v) ethanol and 5 % acetic acid for three 8 minute incubations, and washed in 2.5 % glutaraldehyde for 20 minutes. After rinsing the gel in Milli-Q filtered water, three additional 8 minute washes with water were performed. Following this, the gel was soaked for 20 minutes in DTT (5 $\mu g \cdot L^{-1}$) and 20 minutes in 0.012 M $AgNO_3$. For color development, the gel was rinsed with water for 10 seconds, and then soaked in 0.28 M Na_2CO_3 / 2 mM formaldehyde until bands appeared. The color was fixed by soaking the

gel in 1 % (v/v) acetic acid for 5 minutes. Gel images were captured using an AlphaImager 2000 documentation system with AlphaEase software (Version 4.0; Alpha Innotech Corporation).

Polypeptide size was estimated by comparing relative mobility to that of a set of low molecular weight standards (Sigma). The low molecular weight markers consisted of: BSA (66 kD), fumarase (48.5 kD), carbonic anhydrase (29 kD), and β-lactoglobuline (18.4 kD).

2.5. IMMOBILIZED ALUMINUM ION AFFINITY CHROMATOGRAPHY

Aluminum-binding ability was demonstrated using immobilized metal ion affinity chromatography at 4°C (Basu *et al.* 1997). Chelating Sepharose Fast Flow (Pharmacia) was packed into a 1.5 x 12 cm column (bed volume 13 mL). The metal chelating stationary phase (iminodiacetate (IDA)-agarose) was washed with 250 mL Chelex-purified water (Bio-Rad), and activated with 13 mL of an Al reference solution (Fisher,1000 µg/mL in 4 % HCl). Excess Al was removed by washing with 250 mL Chelex-purified water. The column was equilibrated with 13 mL of 100 mM potassium phthalate (pH 4.0) and 250 µg exudate protein was loaded onto the column and incubated for 2 hours. With the flow-rate determined by gravity, fractions (13 mL) were eluted sequentially with Chelex-purified water, 100 mM potassium phthalate buffer (pH 4.0), 20 mM EDTA (pH 4.0) and 100 mM EDTA (pH 4.0). After each run, the column was regenerated with 75 mL of 100 mM EDTA and 75 mL of Chelex-purified water. In between uses, the column was stored at 4°C in 20 % ethanol.

Fractions from the Al-affinity column were dialyzed using cellulose membranes with a molecular weight cut off of 12– 14 kD. Dialysis was performed overnight against Milli-Q filtered water. Each fraction was then concentrated to 100 µl using the Speed Vac concentrator (Savant SC100). Electrophoretic analysis of 30 µl aliquots from each fraction was performed as described above using SDS-PAGE.

2.6. PROTEIN PURIFICATION AND SEQUENCING

Single step protein purification was performed using the Mini Prep Cell continuous elution system (Bio-Rad). Gel electrophoresis was performed at 4°C under constant current (5 mA). A 4 % polyacrylamide stacking gel (3 cm) and 13 % resolving gel (13 cm) was cast in the gel tube. Concentrated exudate proteins (200 µg) were mixed with an equal volume of sample loading buffer (0.06 M Tris-Cl, pH 6.8; 2 % [w/v] SDS; 25 % [v/v] glycerol; 5 % [v/v] 2-mercaptoethanol; and 0.01 % bromophenol blue) and boiled in a water bath for 3 minutes. Proteins were then loaded onto the tube gel, and electrophoresed for approximately 14 hours. The tank (elution) buffer was the same as used for SDS-PAGE.

Protein fractions (200 µl) were automatically collected from the Mini Prep Cell using a fraction collector (Bio-Rad Model 2100). Collection of fractions started just prior to the elution of the tracking dye. The elution buffer flow rate was 50 $\mu L \cdot min^{-1}$, with 200 µL fractions being collected. To determine which fractions contained the protein of interest, aliquots (10 µL) were analyzed by SDS-PAGE and silver staining as described above. Although a number of bands were isolated using this technique, only the 24 and

40 kD proteins were further processed. Fractions that contained the desired bands were pooled and concentrated under vacuum.

For sequencing, 4 µg and 1.5 µg of the 24 and 40 kD proteins respectively were collected and run on an SDS-PAGE gel. After electrophoresis, the gel was stained with a Coomassie blue solution (0.125 % Coomassie blue R-250, 50 % methanol, and 10 % acetic acid) for 1 hour. De-staining was achieved using two solutions; a single wash of 50 % methanol and 10 % acetic acid for 30 minutes was followed by two washes in 5 % methanol and 7 % acetic acid for 30 minutes each. The 24 kD and 40 kD polypeptide bands were excised from the gel (approximately 40 mm^2 per band) and sent to the Harvard Microchemistry Facility (Harvard University, Cambridge) for micro-sequencing. In-gel digestion was performed on the gel pieces using trypsin. Digested fragments were then separated by HPLC and sequenced by tandem mass spectrometry (MS/MS) on a Finnigan LCQ Quadrupole Ion Trap Mass Spectrometer. One digest fragment from each polypeptide was sequenced. The amino acid sequences obtained were compared to those in the non-redundant GenBank CDS, Translations, PDB, SwissProt, SP update and PIR databases maintained by the National Center for Biotechnology Information. This was achieved using the BLASTP analysis program through the Internet (http://www.ncbi.nlm.nih.gov/cgi-bin/BLAST).

My first attempt at purifying the 24 kD protein was thwarted by loading pre-stained markers on the Mini Prep Cell. Pre-stained markers (Bio-Rad 74177) consisting of phosphorylase B (107 kD), BSA (76 kD), ovalbumin (52 kD), carbonic anhydrase

(36.8 kD), trypsin inhibitor (27.2 kD) and lysozyme (19.2 kD) were mixed with the exudate proteins and loaded on the tube gel. Markers were included to give an estimation of the fractions containing the 24 kD protein. Since the manufacturer states that proteins differing in molecular weight by at least 1 kD can be separated by this technique, the trypsin inhibitor marker (27.2 kD) was not anticipated to interfere with purification. In practice though, my "purified" band (as determined using SDS-PAGE and silver staining) likely contained a small amount of trypsin inhibitor. This contamination became evident when the in-gel trypsin digestion was inhibited. Thus, subsequent purifications through the Mini Prep Cell did *not* include pre-stained markers.

2.7. DETERMINATION OF OXALATE OXIDASE ACTIVITY

Oxalate oxidase catalyzes the formation of hydrogen peroxide and carbon dioxide from oxalate and oxygen. Hydrogen peroxide reacts with a chromatogen (either 4-chloro-1-naphthol or dimethylaniline) to yield a detectable product. Enzymatic assays of oxalate oxidase activity were performed with exudates collected from the sensitive wheat cv. Katepwa and commercially available barley root oxalate oxidase (Sigma O 41278; reconstituted in Milli-Q filtered water). Activity was measured using two methods: histochemical detection and in-gel visualization.

2.7.1. Histochemical detection of oxalate oxidase activity

To localize oxalate oxidase activity along the root, a histochemical staining procedure was used (Dumas *et al.* 1995). After growth in 0 or 100 μM $AlCl_3$ for 72 hours under aseptic conditions, roots were rinsed with Milli-Q (Millipore) water for 5 minutes. Intact roots were placed in a developing solution (25 mM succinate or

potassium phthalate, 3.5 mM EDTA, 2.5 mM oxalic acid, 0.6 mg/mL 4-chloro-1-naphthol; pH 4.0) at room temperature (23°C) until a purple color appeared (approximately 5 minutes). Staining was compared to tissues that were incubated in a developer lacking oxalic acid.

2.7.2. *In-gel detection of oxalate oxidase activity*

To analyze wheat exudates for oxalate oxidase activity, an in-gel detection method was used (Zhang *et al.* 1996). This method isolates oxalate oxidase to a discrete gel region, thereby minimizing the effects of other exudate molecules. Samples of exudate proteins (20 µg) or barley oxalate oxidase (6 µg) were mixed with an equal volume of activity loading buffer (0.06 M Tris-Cl, pH 6.8; 2 % [w/v] SDS; 25 % [v/v] glycerol; and 0.01 % bromophenol blue). The mixtures were loaded without boiling onto a 4 % stacking and 10 % resolving SDS-PAGE gel, and electrophoresed at 10 mA and 20 mA respectively at 4°C. Native oxalate oxidase is stable under the conditions of SDS-PAGE.

For staining, the gel was incubated in gel developing solution (40 mM potassium phthalate, 60 % ethanol, 2 mM oxalic acid, 5 units/mL horseradish peroxidase (Sigma P-8250), and 50 mg / 100mL 4-chloro-1-naphthol) until black bands could be observed (6 to 24 hours). The relative amounts of activity were measured using an AlphaImager 2000 documentation and analysis system (Alpha Innotech Corp.). The spot densitometry tool of the AlphaEase software package was used to calculate the sum of pixel values in each gel lane. The parameters employed were image inversion and automatic background correction to obtain optimized values.

To determine the effect of Al on barley oxalate oxidase activity, 0 – 180 nmol Al was added to 6 µg protein (corresponding to molar ratios of Al to protein from 0 to 3750). Protein was incubated with Al for 10 minutes before activity loading buffer was added. Samples were then loaded, electrophoresed and stained in gel developing solution. To investigate the effect of Al on oxalate oxidase activity in Katepwa exudates, a similar experiment was performed. Because this sample contained a variety of proteins that bind Al, Al could not be added in molar ratios. Instead, Al was added in absolute quantities ranging 0 – 250 nmols Al to 20 µg exudate protein.

The oxalate oxidase assay is dependent on a horseradish peroxidase-mediated conversion of oxalate oxidase-derived H_2O_2 into a color product. To assess the possible interaction of Al with horseradish peroxidase, a control experiment was performed. Aluminum was added to horseradish peroxidase (6 µg) in absolute quantities ranging from 0 – 250 nmol (molar ratios of Al to protein from 0 - 2083). The Al-protein mixture was then incubated for 10 minutes at room temperature. Proteins were mixed with an equal volume of native loading buffer (0.06 M Tris-Cl, pH 6.8; 25 % glycerol; and 0.01% bromophenol blue) and loaded onto a native gel without boiling. Native separation was performed using a 10 % resolving and 4 % stacking gel (as for SDS-PAGE except SDS was omitted). The tank buffer contained 0.025 M Tris (pH 8.3) and 0.192 M glycine, and the gel was electrophoresed at 10 mA and 20 mA through the stacking and resolving gels respectively. Electrophoresis was performed at 4°C.

To stain the horseradish peroxidase gel, the developing solution was identical to the oxalate oxidase developing solution except 1.3 mN H_2O_2 was added as substrate, and horseradish peroxidase was omitted. The conditions of the horseradish peroxidase assay were kept as similar as possible to that of the oxalate oxidase assay; no attempt was made to optimize horseradish peroxidase activity.

2.8. COMPARISON OF EXUDATE PROTEINS FROM AN ALUMINUM SENSITIVE AND AN ALUMINUM TOLERANT WHEAT CULTIVAR

A comparison between the exudate proteins of the Al-sensitive cv. Katepwa and the Al-tolerant cv. Maringa was made. All procedures for collection and analysis of Maringa were identical to those described above for Katepwa.

2.9. EXUDATE PROTEIN PROFILE FOLLOWING SEED EXCISION

To differentiate between the seed and root as sources of exudate protein, a parallel experiment was performed using seedlings after seed excision. Following initial growth in petri dishes, intact seedlings were allowed to establish for 5 days in full nutrient solution (pH 4.3). Subsequently, seeds were gently twisted off, and the seedlings were transferred into fresh full nutrient solution. To eliminate residual seed proteins from the plant, the seedlings were transferred again (after 2 days) into fresh medium. Growth progressed for an additional 7 days before the medium was harvested. Protein content was analyzed only in the exudates collected during this final growth period.

3. RESULTS

3.1. *TRITICUM AESTIVUM* CULTIVAR KATEPWA IS SENSITIVE TO ALUMINUM

Seedlings of *Triticum aestivum* L. cv. Katepwa were shown to be Al-sensitive by monitoring changes in root length and callose deposition of plants grown in full nutrient solution containing 0 - 200 μM $AlCl_3$ over a 72 hour period. Root length was measured at 24-hour intervals for a total of 72 hours (Figure 1A). The greatest change in root length (length (l) minus initial length ($l_{t=0}$)) occurred in the absence of Al. In this control treatment, root length over the 72 hours increased by about 4.4 cm (± SE 0.2). In comparison, root elongation was inhibited in all Al treatments within 24 hours of exposure. When exposed to 50 μM $AlCl_3$, total root length was reduced to 43 % of the control over the 72 hour period. At higher Al concentrations (100 - 200 μM), root elongation was almost completely inhibited. In 100 μM $AlCl_3$ (72 hours), root elongation was less than 32 % of that in control plants. Elongation in 150 μM and 200 μM $AlCl_3$ was only 14 % and 11 % of the control (respectively) over the 72 hour period.

Production of callose (β-1,3-glucan) was used as a second marker of Al sensitivity. Callose production was measured in root tips of plants exposed to a range of Al concentrations for 72 hours (Figure 1B). Little callose was produced in root tips under control conditions. As Al concentrations increased from 50 to 150 μM, the amount of callose increased five-fold. Callose synthesis appeared to level off at higher concentrations of Al (200 μM). Previous studies have shown similar results, illustrating that cv. Katepwa is a sensitive wheat cultivar (Schreiner *et al.* 1994; Basu *et al.* 1994a).

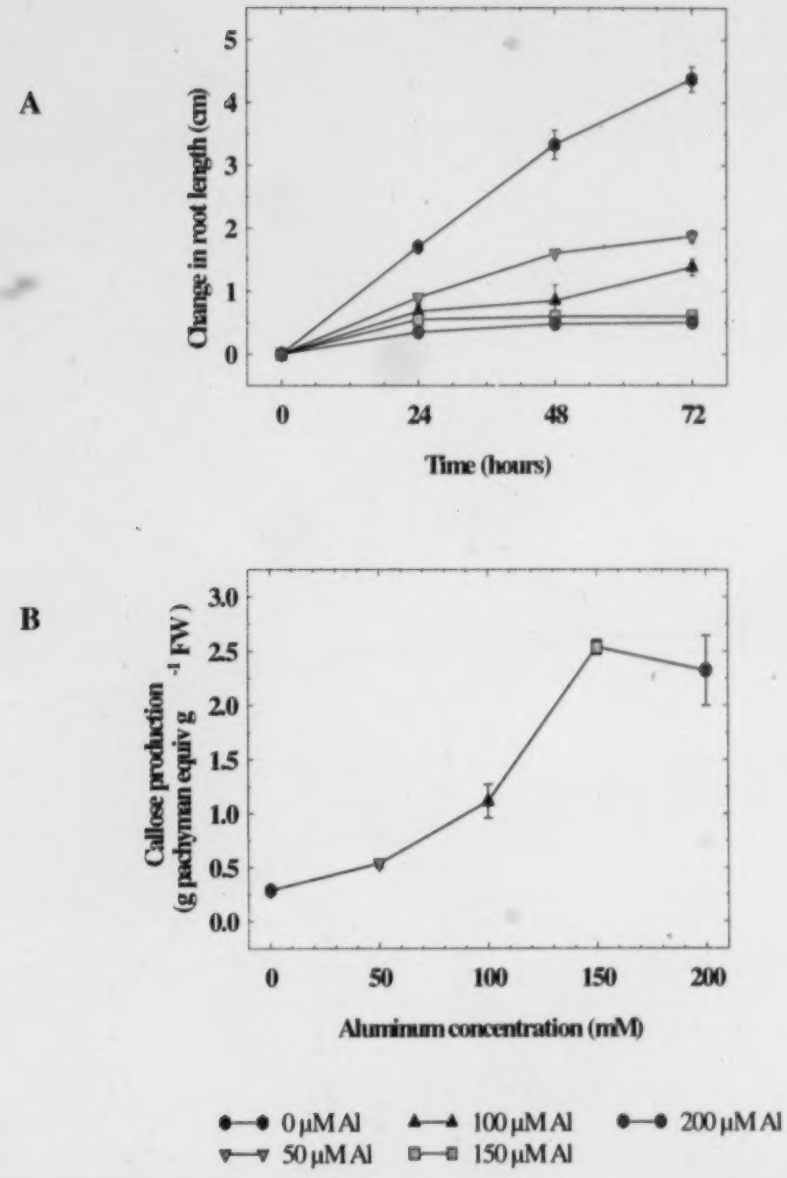

Figure 1. The effect of Al on root elongation (A) and callose production (B) in seedlings of *Triticum aestivum* cv. Katepwa. Roots were exposed to 0 - 200 µM $AlCl_3$ in full nutrient solution (pH 4.3). Root length was measured every 24 hours for 72 hours to estimate changes in root length ($l_t - l_{t=0}$) before root tips were excised and analyzed for callose production. Values represent the means ± SE of three replicates. Aluminum concentrations as low as 50 µM induced symptoms of Al stress.

3.2. THE GROWTH MEDIUM CONTAINS PROTEINS

Exudate proteins from Katepwa were characterized by collecting and concentrating the sterile growth medium bathing seedlings during growth. Proteins in the crude protein mixture were separated by SDS-PAGE and stained for visualization. Protein mixtures typically contained in excess of 14 protein bands (Figure 2). Although some variability was evident in the relative amounts of proteins within the profile, the sizes of the major bands were consistent from one collection to another. Bands with molecular weights of 24 (doublet), 40 (doublet), 45 and 48 kD were usually the most abundant within a profile. The amount of exudate protein collected from the experiments ranged from 17 to 25 μg protein·g root FW^{-1}.

Aluminum treatment affected the number of proteins in the exudate profile (Figure 3). As the concentration of Al increased from 0 to 200 μM, fewer and fewer bands were detected. In the presence of 100 μM $AlCl_3$, only half of the proteins present in the control exudates were still evident. When the Al concentration was increased to 200 μM, only the 24 and 40 kD bands remained clearly detectable. Interestingly, the 24 kD band appeared to increase in intensity under mild Al treatment (100 to 150 μM), while the 40 kD band decreased. Also evident was the induction of 22 and 27 kD bands in the 100 and 150 μM Al solutions.

3.3. THE 24 AND 40 KD EXUDATE PROTEINS BIND ALUMINUM

Immobilized metal affinity chromatography was used to determine whether exudate proteins are capable of binding Al. Binding with Al would suggest that a

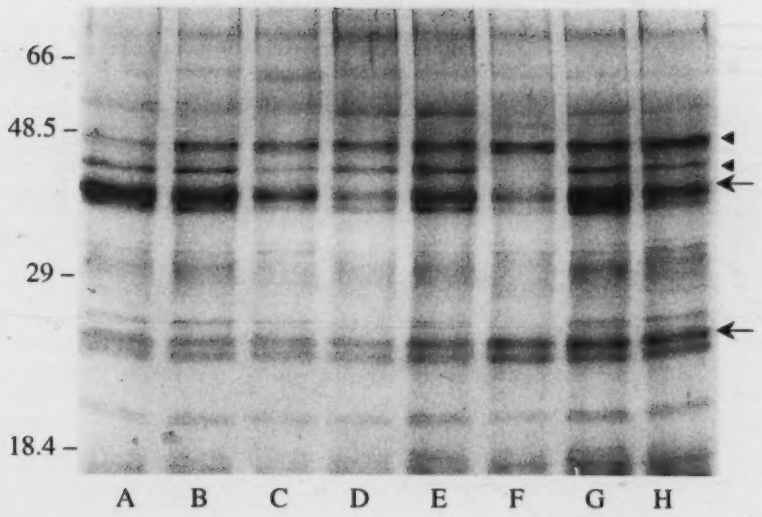

Figure 2. Comparison of exudate protein profiles from seedlings of cv. Katepwa harvested from independent experiments (Lanes A-H). Seedlings were surface sterilized with $HgCl_2$, germinated on enriched agar, and then grown in nutrient solution for 7 days. Proteins in the growth medium were tested for sterility and then concentrated. Ten micrograms of protein were loaded onto each lane. Molecular weights are indicated at the left in kD. Arrows indicate proteins subsequently purified. Arrowheads indicate the molecular weights of other major bands. Constitutive proteins are similar throughout all experiments.

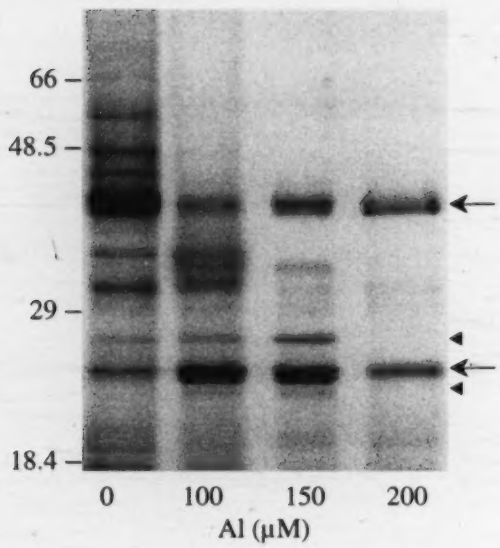

Figure 3. SDS-PAGE analysis of exudate proteins from seedlings of cv. Katepwa exposed to increasing concentrations of Al during growth. Proteins were collected from the growth medium after 7 days. Numbered lanes represent Al concentration (μM). Each lane contains 6 μg of protein, and bands were detected by silver staining. Molecular weight markers in kD are indicated at the left. Arrows indicate the proteins subsequently purified. Arrowhead indicates 22 and 27 kD bands induced in 100 and 150 μM Al. Aluminum treatment results in a reduction in the number of detectable exudate proteins.

specific protein might represent a potential site for Al-induced injury. Sequential washes of the immobilized metal ion affinity column were compared to the total exudate protein profile (Lane A) using SDS-PAGE (Figure 4). The first two washes with water (Lane B) and 100 mM potassium phthalate (Lane C) removed excess and nonspecifically-bound proteins. Bands present in Lane B suggest that the column may be overloaded with protein. The absence of bands in Lane C confirms that the water wash removed nearly all excess proteins. The ionic strength of the wash buffer (100 mM potassium phthalate) ensures that non-specifically bound proteins are washed off by bulk flow. The last two washes (Lane D, 20 mM EDTA; Lane E, 100 mM EDTA) removed the Al-protein complexes by competing for Al-binding sites on the stationary phase (IDA-sepharose). The low EDTA wash removes weakly bound proteins, while the high EDTA concentration washes off tightly adhering proteins. The protein profiles of these two washes were nearly identical, although a larger proportion of total protein washed off in the high EDTA wash. This suggests that most of the exudate proteins bind to Al strongly. Only one band of approximately 45 kD is absent from the EDTA washes. Since this band is evident in the water wash, it likely does not have any Al-binding affinity under the elution conditions. When the column is not activated with Al, all of the proteins washed off in the water fraction (data not shown).

The 24 and 40 kD proteins were chosen for purification based on four observations: 1) they are constitutively expressed, suggesting they may have a biological function under non-stress conditions; 2) they continue to be exuded during Al exposure, suggesting they may be of central importance to seedling growth during stress; 3) they

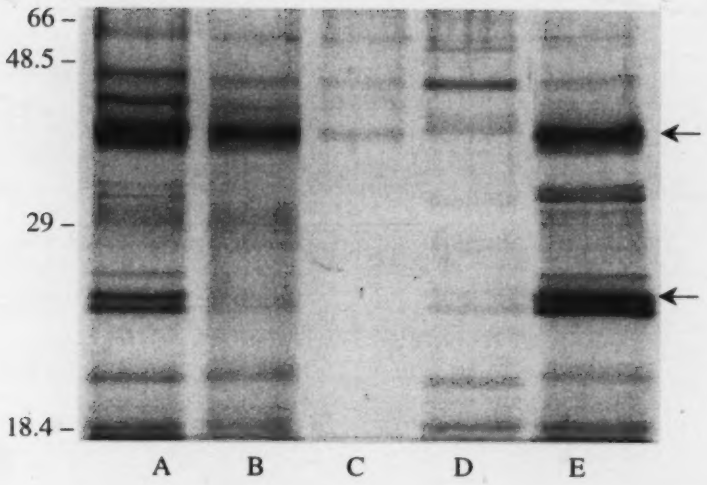

Figure 4. Analysis of exudate proteins using immobilized Al affinity chromatography. Exudates were collected from the medium (pH 4.3) surrounding the roots of Katepwa seedlings after 7 days of growth. Different lanes represent proteins washed off in sequential fractions. Equal volumes of each fraction were loaded onto the gel. Lane A, total root exudates (6 µg); B, water; C, 100 mM potassium phthalate (pH 4.0); D, 20 mM EDTA (pH 4.0); E, 100 mM EDTA (pH 4.0). Molecular weight markers in kD are indicated at the left. Arrows indicate the proteins subsequently purified. Most exudate proteins bind to Al.

bind to Al relatively strongly, suggesting activity could be directly inhibited by Al; and 4) they are the major proteins in the protein profile, easing the process of protein purification.

3.4. PURIFICATION, SEQUENCING AND HOMOLOGY SEARCHES REVEAL HOMOLOGY WITH GERMIN-OXALATE OXIDASE AND SERPIN

In order to identify putative protein functions, the 24 and 40 kD proteins were purified by continuous flow gel electrophoresis (Figure 5). SDS-polyacrylamide gels of sequential fractions collected during the separation of crude exudates were used to identify fractions corresponding to the 24 and 40 kD exudate proteins. The 24 kD protein was isolated from fraction 141, and the 40 kD protein was isolated from fractions 106 to 110. These bands were purified to homogeneity using this single electrophoretic procedure, but fractions were pooled from independent purifications to obtain sufficient protein (Figure 6).

Once enough protein was collected, they were sent for sequencing. Sequences of 16 and 14 amino acids were obtained for the 24 kD (Figure 7) and 40 kD (Figure 8) bands respectively (Table 1 for summary of sequence data). Homology searches suggest the proteins have been previously identified in wheat. The 24 kD protein appeared to be one of the subunits of germin-oxalate oxidase, while the 40 kD protein appeared to be a serine proteinase inhibitor (serpin). Although the 24 kD fragment showed 100 % identity with several oxalate oxidases, only the first three identified through the database search are presented. These included a barley oxalate oxidase induced in leaves by fungal infection (Zhou *et al.* 1998), and two wheat germins associated with seed germination

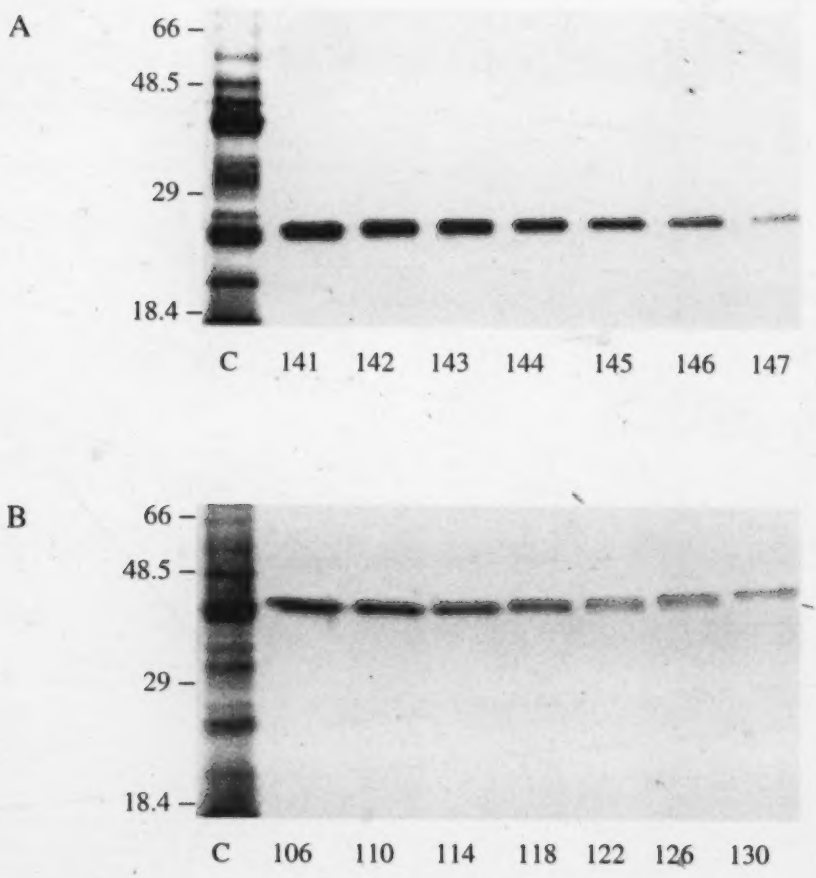

Figure 5. SDS-PAGE analysis of selected protein fractions collected by continuous flow gel electrophoresis. Fractions in (A) were collected from a separate electrophoretic run than in (B). Lane C (crude) contains 3 µg of exudate proteins. Numbers represent the fraction collected. Fraction 141 was used for sequencing the 24 kD polypeptide and fractions 106 to 114 were pooled and used for sequencing the 40 kD polypeptide. Molecular weight markers in kD are indicated at the left.

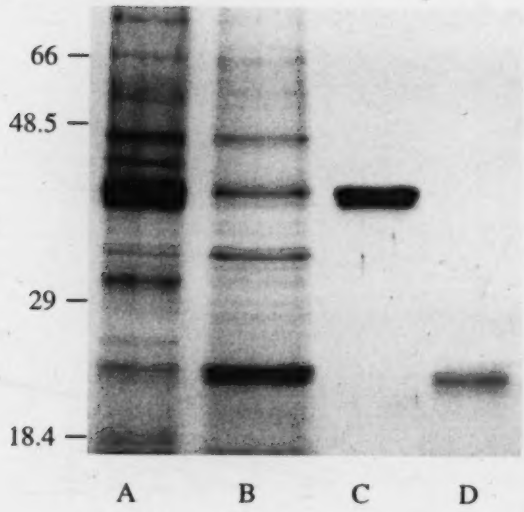

Figure 6. SDS-PAGE summary of aluminum-binding exudate proteins and the purified polypeptides. Lane A, total exudate proteins (10 µg); Lane B, aluminum-binding exudate proteins; Lane C, purified 24 kD polypeptide; Lane D, purified 40 kD polypeptide. Peptides were purified through continuous flow gel electrophoresis. Proteins were visualized by silver staining. Molecular mass markers in kD are indicated at left.

```
BOxO  (Y14203) mgysknlgag lftmlllapa imatdpdplq dfcvadldgk avsvnghtck pmseagddfl      60
WGerm (Y09917) ********** **g******g vl*s******* ********** *******p** **********      60
WGerm (P26759) ******ias* m*a*****s* vlssn*h*** ********** *******m** **********      60

24 kD                                                  v dfapggtnpp hihpr
BOxO  (Y14203) fsskltkagn tstpngsavt eldvaewpgt ntlgvsmnrv dfapggtnpp hihprateig  120
WGerm (Y09917) *****a**** ********** ********** ********** ********** ********d**  120
WGerm (P26759) *****a**** ********** d*n******* *********** ********** **********  120

BOxO  (Y14203) mvmkgellvg ilgsfdsgnk lysrvvrage tfviprglmh fqfnvgktea ymvvsfnsqn  180
WGerm (Y09917) i*l******* ****l***** ********** **l******* ********h* s*********  180
WGerm (P26759) i********** ****l***** ********** **l******* ********** s***f****s  180

BOxO  (Y14203) pgivfvpltl fgsnppiptp vltkalrvea gvvellkskf aggs
WGerm (Y09917) ******ltlf ********** ********** ********n* ****
WGerm (P26759) *sv******* ********k* ********** ********** ****
```

Figure 7. Multiple sequence alignment of the 24 kD polypeptide with related proteins A 16 amino acid sequence was obtained after trypsin digestion and sequencing by tandem mass spectrometry. BOxO (accession no. Y14203) is a barley (*Hordeum vulgare*) oxalate oxidase involved with pathogen response (Zhou *et al.* 1998); WGerm (Y09917; Bashiardes and Cuming 1996) and WGerm (P26759; Lane *et al.* 1991) are wheat germination-related proteins. Amino acid residues identical to BOxO (Y14203) are indicated with asterisks (*). Sequence identity with the 24 kD protein is bolded. The 16 amino acids of the 24 kD tryptic fragment shares 100 % identity with these oxalate oxidases.

```
WS (Y11486) mattlatdvr lsiahqtrfa frlasaissn pestvnnaaf spvslhvals litagaggat      60
WS (Y11485) ********** ********** l****t**** *k*aas**** ******s*** *la****s**      60
WS (Z49890) ********** ********** l****t**** *k*aas**** *****ys*** *la****s**      60

WS (Y11486) rnqlaatlge geveglhala eqvvqfvlad asniggprva fangvfvdas lqlkpsfqel     120
WS (Y11485) *d**v****t *****g**** ********** **sa****** ********** *l********     120
WS (Z49890) *d**v****t *k******** ********** **st**sacr ********** *l*******I     120

WS (Y11486) avckykaeaq svdfqtkaae vtaqvnswve kvttglikdi lpagsidntt rlvlgnalyf     180
WS (Y11485) *********t* ********** **t******* ***s*r**n* **s**v**** k***a*****     180
WS (Z49890) *********t* ********** **t******* ***s*r**** **p******* k***a*****     180

WS (Y11486) kgawtdqfdp ratqsddfyl ldgssiqtpf myssee-qyi sssdglkvlk lpykqggdkr     240
WS (Y11485) *********s yg*kn*y*** *****v**** *s*mddd*** ********** ********n*     240
WS (Z49890) *****e***s yg*kn*y*** *****v**** *s*mdd-**l l********* ********n*     240

40 kD                          lsaepefle qhipr
WS (Y11486) qfsmyillpe alsglwslae klsaepefle qhiprqkval rqfklpkfki slgieasdll 300
WS (Y11485) ********** *pg**s**** ******d*** r*****r**i ********** *f*m****** 300
WS (Z49890) **f******* *pg**s**** ******d*** r*****r*** ********** *f******** 300

WS (Y11486) kglglllpfg aeadlsemvd spmaqnlyis sifhkafvev netgteaaat tiakvvlrqa     360
WS (Y11485) *c***q***s d***f***** ***p*g*rv* *v**q***** **q******s *ai*m*pq**     360
WS (Z49890) *c***q**** d***f***** *l*p*g*rv* *v**q***** **q******s *ai*m**q**     360
```

Figure 8. Multiple sequence alignment of the 40 kD polypeptide with related proteins A 14 amino acid sequence was obtained after trypsin digestion and sequencing by tandem mass spectrometry. All sequences are wheat (*Triticum aestivum*) serine protease inhibitors (serpins) involved with germination. WS (accession no.Y11486, SK Rasmussen 1997) is a serpin with a putative trypsin inhibitory reactive site, WS (Y11485, SK Rasmussen 1997) is a natural mutant of wheat serpin, and WS (Z49890, Rasmussen *et al.* 1996) is a recombinant serpin with inhibitory activity. A gap (-) at residue 217 is introduced in WS (Z49890) to maximize similarity. Amino acid residues identical to WS (Y11486) are indicated with asterisks (*). Sequence identity with the 40 kD protein is bolded. The 14 amino acids of the 40 kD tryptic fragment shares 86 % identity with these serpins.

Table 1. Summary of sequence data obtained for two wheat exudate proteins.

Band size (kD)	Peptide mass	Sequence determined by tandem mass spectrometry	Protein identified by NCBI databases
24	1710.87	VDFAPGGTNPPHIHPR	Oxalate oxidase
40	1664.86	LSAEPEFLEQHIPR	Serine proteinase inhibitor

(Bashiardes and Cuming 1996; Lane *et al.* 1991). The 40 kD fragment shared 100 % identity with a wheat serpin associated with seed germination (Rasmussen 1997); and 86% identity with a natural serpin mutant (Rasmussen 1997) and recombinant serpin (Rasmussen *et al.* 1996). The 40 kD protein (a putative serine proteinase inhibitor) did not appear to have inhibitory activity against trypsin (a serine proteinase) under the conditions of the in-gel digest. No difficulty was experienced with this reaction prior to sequencing.

Since oxalate oxidase has been associated with a variety of plant stress responses, including pathogen (e.g. Zhou *et al.* 1998), osmotic (e.g. Hurkman and Tanaka 1996a) and metal stress (e.g. Hamel *et al.* 1996), the remainder of this project focused on the effect of Al on oxalate oxidase activity.

3.5. OXALATE OXIDASE ACTIVITY IS LOCALIZED ALONG INTACT ROOTS

The distribution of oxalate oxidase activity was visualized *in situ* using a histochemical staining method. Seedlings of Al-sensitive cv. Katepwa and Al-tolerant cv. Maringa were assayed after growth in the presence or absence of 100 μM $AlCl_3$ for 72 hours (Figure 9). Staining was shown to be specific for oxalate oxidase activity because color development did not occur in the absence of oxalate.

When roots of control seedlings (0 μM $AlCl_3$) were stained, oxalate oxidase activity was readily observed in both primary and lateral roots of both cultivars. Activity

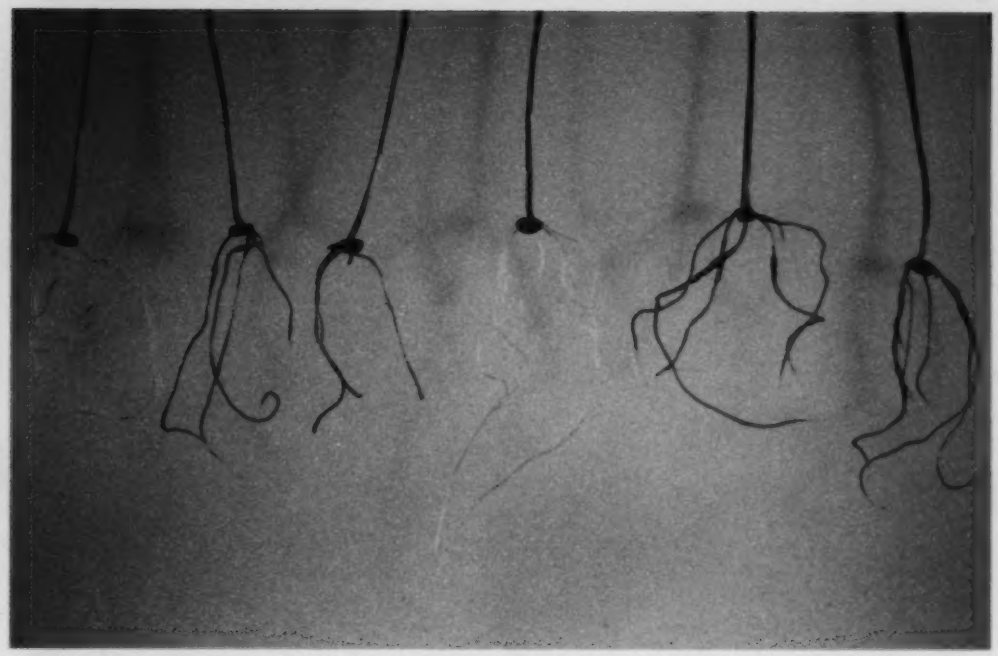

Figure 9. Detection of oxalate oxidase activity along roots of Al-sensitive Katepwa (K) and Al-tolerant Maringa (M) cultivars of *Triticum aestivum*. Roots were exposed to 0 (-Al) or 100 (+Al) μM $AlCl_3$ in nutrient solution (pH 4.3) for 72 hours under sterile conditions. Roots were then rinsed in water for 5 minutes before staining. The developing solution contained 25 mM potassium phthalate (pH 4.0), 3.5 mM EDTA, 2.5 mM oxalate and 0.6 mg / mL 4-chloro-1-naphthol. For comparison, control roots were also stained in developer without oxalate (-ox). Oxalate oxidase staining is localized to mature root regions in all treatments except when Katepwa is grown in Al.

was restricted to the mature root regions. No staining was evident at the root tips. There was no discernable difference in the staining intensity between cultivars. A difference between cultivars only became apparent after plants were grown in 100 μM $AlCl_3$. Roots of cv. Maringa (Al-tolerant) grown in Al stained for activity only in the mature root regions. This Al-tolerant cultivar exhibited the same staining pattern, regardless of Al treatment. Cultivar Katepwa, however, differed in the staining pattern with Al treatment. In addition to the classic symptoms of root stunting and lateral root inhibition, Al treatment produced uniform activity staining along the whole root length. Both mature root regions and the root tip became intensely stained under Al stress.

Although this assay provides information on the site of enzyme activity along roots, it does not provide quantitative data regarding possible Al-induced enzyme inhibition. Using this procedure, staining intensity was similar in both cultivars whether grown in the presence or absence of 100 μM $AlCl_3$. Consequently, an alternative method was used to determine whether Al inhibits the activity of this enzyme.

3.6. IN-GEL STAINING DETECTS ALUMINUM-INDUCED INHIBITION OF OXALATE OXIDASE ACTIVITY IN EXUDATES

To directly determine whether Al inhibits wheat exudate oxalate oxidase, activity was detected in-gel following SDS-PAGE. Commercial barley oxalate oxidase was included for comparison. Two bands were observed after staining for activity in control exudates (Figure 10). The upper band co-migrated with a commercial preparation of barley oxalate oxidase (125 kD) while the second band migrated slightly ahead. Staining was consistently more intense for the second band.

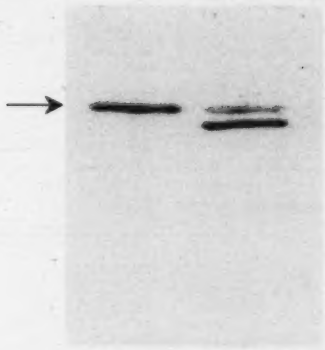

Figure 10. Comparison of oxalate oxidase activity between commercially-available barley (*Hordeum vulgare*) and wheat (*Triticum aestivum*) cv. Katepwa exudates. Four micrograms of barley oxalate oxidase and 20 µg of wheat exudate proteins were loaded (without boiling) onto a SDS-polyacrylamide gel. Bands of activity were stained in a solution containing 40 mM potassium phthalate (pH 4.0), 60 % ethanol, 2 mM oxalate, 5 units / mL horseradish peroxidase, and 50 mg / 100mL 4-chloro-1-naphthol. The arrow indicates a molecular weight of 125 kD. Katepwa exudates contain activity similar to the barley oxalate oxidase.

To determine whether Al influences oxalate oxidase activity, exudates were directly incubated in Al. Barley oxalate oxidase activity was clearly inhibited by an Al: protein molar ratio of 1250 (Figure 11A). Densitometry scanning demonstrated that only 64 % of control activity remained under such conditions. As the amount of Al was increased above a ratio of 1250, activity decreased further. Activity was almost completely inhibited at a ratio of 3750, where only 29 % of control activity was detectable. Equal protein loading was verified in each of these treatments using silver staining (Figure 11A). The ratio of activity staining to silver staining generally decreased with increasing Al (values in order of increasing Al: 1.00, 1.05, 0.96, 0.65, 0.49 and 0.33). When wheat exudates were examined for activity, both isoforms were relatively unaffected by amounts of Al up to 50 nmol (Figure 11B). By 150 nmol Al, only trace levels of activity were detectable (29 % of control activity by densitometry). Very little activity could be detected in the presence of 200 nmol Al or greater. Once again, silver staining showed that nearly equal protein loading was achieved with low levels of Al (up to 150 nmol Al; Figure 11B), while higher amounts resulted in less protein. At these higher concentrations, a visible precipitation of the sample was observed to remain in the gel loading well, possibly explaining the decrease of protein detected by silver staining. Again, the ratios of activity staining to silver staining generally decreased with increasing Al (values in order of increasing Al: 1.00, 0.98, 0.94, 0.51, 0.30, 0.26, and 0.31). To confirm that the oxalate oxidase inhibition was not explained by an effect on horseradish peroxidase, the effect of Al on this enzyme was assayed. In contrast to the oxalate oxidase response, Al did not inhibit horseradish peroxidase (Figure 11C). This

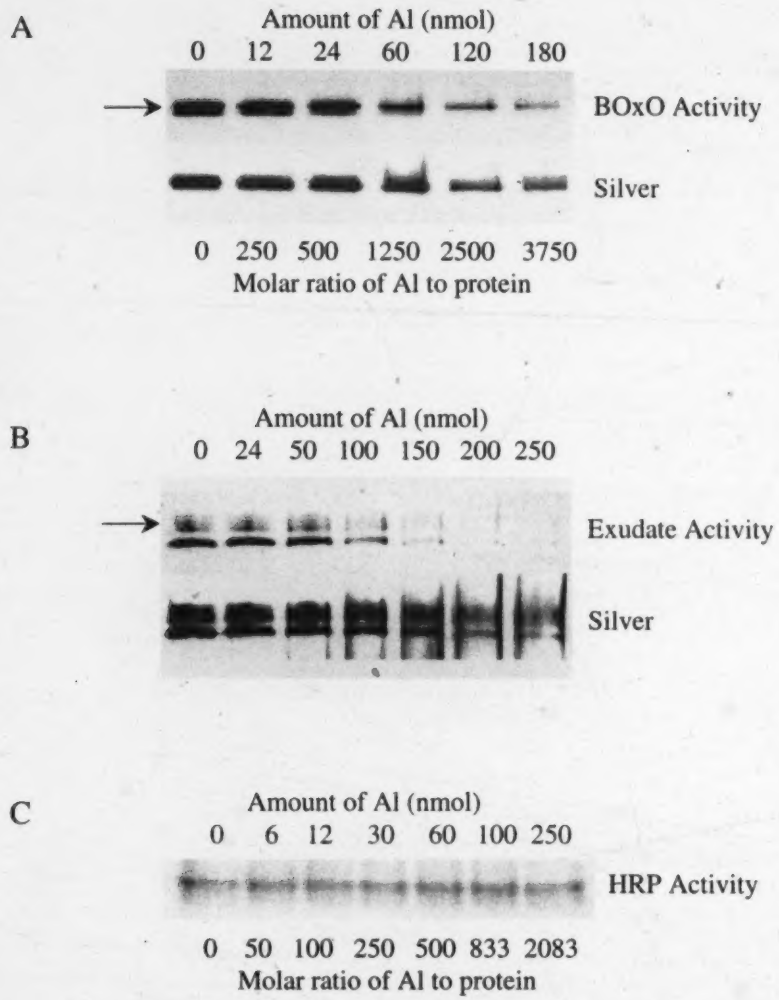

Figure 11. The effect of Al on oxalate oxidase activity of a barley protein and wheat exudate proteins. (A) The activity of barley oxalate oxidase (BOxO) was assayed in the presence of 0 - 180 nmol Al (0 - 3750 Al to protein molar ratios), and the same gel was then silver stained. (B) The activity of oxalate oxidase of wheat exudates was assayed in the presence of 0 - 250 nmol $AlCl_3$ and then silver stained. (C) The effect of Al on horseradish peroxidase (HRP) was assayed in the presence of 0 - 250 nmol Al (0 - 2083 Al to protein molar ratios). The arrows represent a molecular weight of 125 kD. Aluminum inhibited the activity of oxalate oxidase.

differential inhibition also suggests that Al inhibition is specific to the enzyme tested.

It is difficult to directly compare the amount of Al needed to inhibit barley and wheat oxalate oxidases. This is due to the nature of the enzyme samples. Barley oxalate oxidase was purchased as a partially purified powder, while wheat oxalate oxidase was only available as a crude exudate preparation. The wheat exudate also contained two isoforms, and a number of other proteins, which bind to Al.

3.7. ALUMINUM-SENSITIVE AND ALUMINUM-TOLERANT CULTIVARS HAVE SIMILAR EXUDATE PROTEIN PROFILES

To determine whether the exudate protein profiles are similar between cultivars, a comparison was made between the Al-sensitive cv. Katepwa and the Al-tolerant cultivar Maringa. As mentioned previously, active oxalate oxidase was present along Maringa roots and remained localized to mature root regions under 100 µm Al exposure (Figure 9). The 24 and 40 kD peptides were both abundant in Maringa exudates (Figure 12A). Two active isoforms of oxalate oxidase were also detectable in Maringa exudates using the in-gel detection method (Figure 12B). The protein profile of Maringa is essentially identical to that of Katepwa, although band intensity varied relative to other bands within the profile in comparison to Katepwa.

3.8. SEED EXCISION ELIMINATES HALF THE EXUDATE PROTEINS FROM THE GROWTH MEDIUM, INCLUDING THE 24 AND 40 KD PROTEINS

Proteins present in the growth medium have at least two possible tissue origins, roots and seeds (Figure 13). When seeds were excised from the seedling, only about half

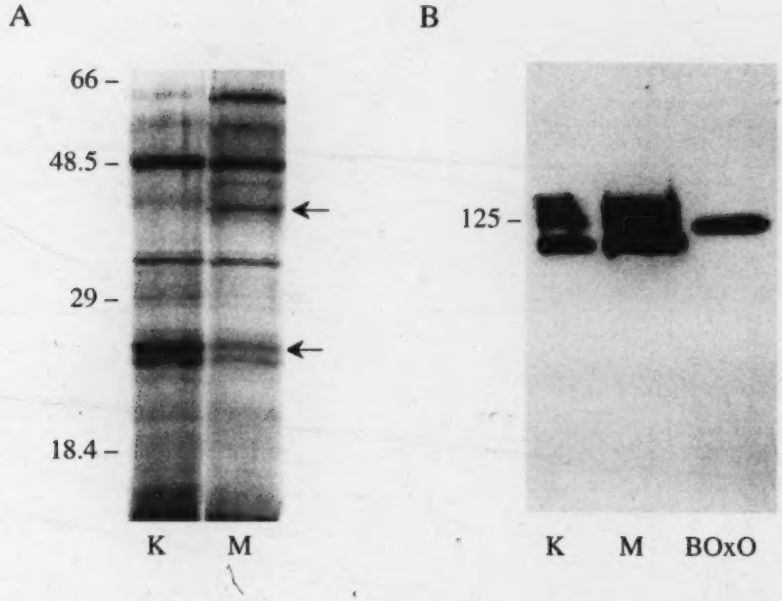

Figure 12. Comparison of exudate protein profiles (A) and oxalate oxidase activity (B) between Al-sensitive Katepwa (K) and Al-tolerant Maringa (M) wheat cultivars. Exudate proteins were collected from the growth medium during 7 days of growth in a nutrient solution (pH 4.3). Ten micrograms of exudate protein were loaded and then detected with silver staining (A). Twenty micrograms of exudate protein were loaded and then detected with oxalate oxidase developing solution (B). Six micrograms of barley oxalate oxidase (BOxO) were loaded for comparison in B. Molecular weight markers in kD are indicated at left. Arrows indicate the 24 and 40 kD proteins purified. Oxalate oxidase was present in both Katepwa and Maringa exudates.

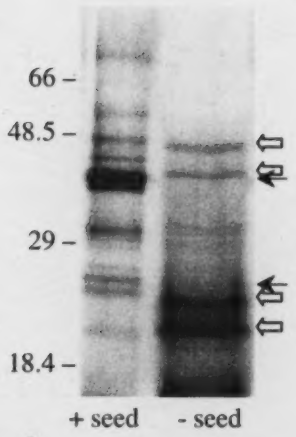

Figure 13. SDS-PAGE comparison of exudate proteins from seedlings of cv. Katepwa in the presence (+) and absence (-) of the seed. Proteins were collected from the growth medium after 7 days. Each lane contains 3 µg protein, and bands were detected by silver staining. Molecular weight markers in kD are indicated at the left. Filled arrows indicate the 24 and 40 kd purified proteins, and hollow arrows indicate the dominant root exudate proteins. The 24 and 40 kD proteins both originate in the seed.

the number of proteins was detected in the growth medium using silver staining (Figure 13). Both the 24 and 40 kD proteins appear to originate from the seed, since neither can be detected following seed excision (Lane B). Furthermore, when assayed for oxalate oxidase, exudates collected in the absence of seeds tested negatively for activity (data not shown). The major bands that appear to originate from the root correspond to molecular weights of 22.8, 23.5, 41, and 45 kD.

4. DISCUSSION

4.1. GENERAL DISCUSSION

Aluminum induces a wide range of lesions in plants. Many of these injuries are localized at the cell wall and plasma membrane. Since Al induces a concert of dysfunctions, the present study set out to identify potential new sites of injury in the apoplasm/rhizosphere. To this end, two exudate proteins were purified from the growth medium of the Al-sensitive wheat cultivar Katepwa. The culmination of my work suggests that oxalate oxidase may represent a target of Al injury.

Triticum aestivum L. cv. Katepwa can be found in agricultural fields across Canada. It was chosen for the present experiments because of its prevalence in the field and Al-sensitive nature. In searching for Al-induced lesions, a sensitive cultivar would be more appropriate since it likely expresses fewer (or lacks certain) tolerance mechanisms. Consequently, sensitive sites in Al-sensitive cultivars would be more vulnerable to injury than those in Al-tolerant cultivars and also easier to detect under lower concentrations of Al. Previous reports have established that cv. Katepwa is Al-sensitive relative to other cultivars using root elongation and callose deposition as markers of injury (Basu *et al.* 1994a; Schreiner *et al.* 1994). The present study further supports this classification using these indicators. Aluminum concentrations as low as 50 μM induced root stunting and callose deposition in root tips (Figure 1). Treatment with 100 μM Al was sufficient to nearly prevent root elongation and triple callose production.

I identified a suite of proteins in the growth media of Katepwa seedlings. These proteins probably consist of "free" rhizosphere proteins and apoplasmic proteins loosely-

associated with the cell wall since they were isolated from the bulk solution surrounding roots. My control protein profile (Figure 2) is similar overall to the banding pattern reported by Basu *et al.* (1994a). In the presence of Al, however, I detected fewer and fewer proteins, with nearly all proteins disappearing in the presence of 200 μM Al (Figure 3). This is in contrast with Basu *et al.* (1994a) who noted that only a few exudate proteins disappeared from the protein profile as the concentration of Al increased to 200 μM. This discrepancy may be due to differences in protein loading; I loaded a smaller amount of protein, possibly resulting in my detection of only the most dominant bands. The loss of bands in the presence of Al may be due to two factors: 1) Al may accelerate the degradation of proteins already in the rhizosphere, or 2) Al may inhibit the synthesis of, or exudation of, proteins into the rhizosphere. Regardless of this discrepancy, the 24 and 40 kD bands were abundant and expressed both in the presence and absence of Al in both studies (Basu *et al.* 1994a; present study).

In comparing exudate proteins from the Al-sensitive (Katepwa) and the Al-tolerant (Maringa) cultivars, I detected no major differences in the protein profiles (Figure 12A). This is in accordance with Basu *et al.*'s study (1994a), where differences between cultivars could only be detected following Al exposure. Treatment with Al resulted in the induction of three exudate proteins in Maringa that were not detectable in Katepwa (Basu *et al.* 1994a). From their work, no proteins appeared to be induced in Katepwa. In contrast, my experiment showed that two proteins (22 and 27 kD) were induced by 100 and 150 μM Al (Figure 3). The identity of these proteins is unknown.

To date, many biologically important enzymes have been identified in the rhizosphere (see Introduction). In the present study, at least 14 constitutive protein bands were detected in the growth medium of seedlings grown under non-stress conditions (Figure 2). Each represents a potential target of Al. Two proteins were purified from the nutrient medium surrounding the roots of Katepwa. These proteins were chosen for purification based on three prerequisites. Firstly, the proteins had to be expressed under non-stress conditions. This requirement eliminated proteins that are specifically induced by Al. Such proteins have been proposed to have a role in metal detoxification (Puterril and Gardner 1988), and were consequently of little interest to my project. Constitutive enzymes are more likely targets that, upon binding with Al, could result in symptoms of Al stress. The second prerequisite for selecting proteins was their presence in the apoplasm/rhizosphere under conditions of Al stress. Aluminum has been shown to reduce the number of proteins in the growth medium of Katepwa (Basu *et al.* 1994a). If the proteins of interest were present under physiologically relevant Al concentrations (~100 μM $AlCl_3$), their activity could be inhibited upon binding with Al. Only about half the number of proteins collected under non-stressed conditions continued to be detectable in the presence of 100 μM $AlCl_3$ (Figure 3). Finally, the third prerequisite for purification was that the proteins must have the ability to form a stable complex with Al. This criterion selects for proteins that could be inhibited directly upon binding with Al. Calmodulin, for example, loses its helical structure upon binding with Al. As a result, it loses its regulatory activity (Siegel and Haug 1983). Immobilized Al ion affinity chromatography was used to show that nearly all Katepwa exudate proteins bind with Al (Figure 4).

Based on these prerequisites and their relative abundance in the protein profile, the most suitable proteins for purification were the 24 and 40 kD proteins.
Protein purification was achieved using continuous flow gel electrophoresis. Although purification was accomplished by a single procedure, collection of sufficient protein for micro-sequencing was a major undertaking (see section 4.5). This achievement was further exacerbated by the initial inclusion of pre-stained markers. Thus, although my protein purification procedure appears simple, it was in fact a formidable task.

4.2. SERINE PROTEINASE INHIBITORS

One of the proteins purified and identified was a 40 kD exudate protein that binds strongly to Al. Amino acid sequence information showed homology to serine proteinase inhibitors (serpins). Serpins are found across a diverse range of organisms including mammals, insects, viruses, and plants (Marshall 1993). All share structural similarity in amino acid sequence and overall tertiary structure (Marshall 1993). As their name suggests, most serpins have been shown to have inhibitory activity. In mammals, serpins are involved in the regulation of important physiological processes such as blood coagulation, hormone transport, and blood pressure maintenance (Whisstock *et al.* 1998). Some serpins like HSP47 acts as chaperones involved with protein folding, while ovalbumin acts as a storage protein (Whisstock *et al.* 1998). Comparatively, little is known about the function of serpins in plants. Serpin isoforms have only been identified from the endosperm of barley and wheat grains (Hejgaard *et al.* 1985; Rosenkrands *et al.* 1994). Although they constitute a major proportion of the seed albumins, their specific biological function is uncertain. Serpins exist in both soluble and bound forms, which become released during germination (Hejgaard 1976). Only certain serpins have been

shown to have inhibitory activities against chymotrypsin and trypsin. This function is thought to be involved in regulating protein degradation during seed germination (Dahl *et al.* 1996). Plant serpins without known inhibitory activity have been proposed to function as storage proteins or defence enzymes directed against the digestive proteinases of insect pests (Dahl *et al.* 1996).

I identified a putative serpin in the exudates of cv. Katepwa. The 40 kD band was no longer detectable in exudates after seed excision (Figure 13). This suggests the wheat serpin originated from the seed, an observation that is consistent with the germination-related function that has been proposed for serpins. Although the 40 kD protein binds strongly to Al, my data does not provide evidence for a direct involvement in the Al toxicity syndrome. This is based on its seed origin and the observation that Al appears to have little effect on germination (de Lima and Copeland 1990). In their study, de Lima and Copeland found that Al only inhibits root elongation after seeds are already germinated. They detected no difference in the number of seeds that germinate in the presence and absence of Al. The binding of seed-borne proteins to Al may be coincidental in this case, thus likely contributing little to root injury. Given the lack of information about its role in roots and the difficulty in assaying for enzyme activity, no studies were pursued with this protein. Nonetheless, this does not dismiss a possible interaction between Al and serpin during germination and early seedling establishment.

4.3. GERMIN-OXALATE OXIDASE

Results from my sequence analysis suggest that the 24 kD protein is a subunit of germin-oxalate oxidase. Structurally, germins are thought to either be a homopentamer

or a trimer of dimers (Gane *et al.* 1998). Functionally, germins appear to be involved in developmental and stress-related processes. Although it is not known whether all germin genes are regulated by both factors, the germin gf 2.8 gene has recently been shown to be regulated by time and certain biotic and abiotic stresses including metals, polyamines, wounding and viral infection (Berna and Bernier 1999). Germins have been identified throughout the plant in leaves, roots, mature grains, immature seeds (pseudogermin), flowers and fruit (Membre *et al.* 1997). Although germin-like proteins have been identified in *Arabidopsis* (Membre *et al.* 1997), lupin (Wojtaszek *et al.* 1998), canola (Membre *et al.* 1997), pine (Domon *et al.* 1995), and the slime mold *Physarum* (Bernier *et al.* 1987), only wheat and barley isoforms have demonstrated oxalate oxidase activity (oxalate:oxygen oxidoreductase, EC 1.2.3.4.) (Lane *et al.* 1993; Dumas *et al.* 1993). Oxalate oxidase catalyzes the conversion of oxalate into H_2O_2 and CO_2. The generation of hydrogen peroxide is thought to be important in regulating many cellular processes including: signal transduction (Apostol *et al.* 1989; Bradley *et al.* 1992), peroxidase-mediated cross-linkage of cell wall polymers (Dunwell 1998), induction of programmed cell death (Levine *et al.* 1994), lignification (Dunwell 1998) and, by combining with chloride to yield hypochlorite, resistance to pathogens (Lane 1994). Since oxalate is stored in plant cells as a calcium salt, Ca^{2+} is also released through the activity of this enzyme. Calcium ions function as secondary messengers in a variety of hormonal and environmental responses and also play a pivotal role the regulation of cellular homeostasis (Rengel 1992).

Germin was first studied as the protein marking the onset of germination in wheat (Grzelczak and Lane 1984). Its subunits show unusual resistance to dissociation by heat, SDS, extreme pH, and proteinases (Grzelczak and Lane 1984; Lane 1994). Cell wall-associated germin accounts for about 40 % of the total germin in germinating embryos, while the other half is of unknown cytoplasmic origin (Lane *et al.* 1986; Lane *et al.* 1992). Germin in the cell wall is primarily associated with highly-substituted forms of glucuronoarabinoxylans, which have been strongly implicated in cell wall expansion (Gibeaut and Carpita 1991). Apoplastic germin is so tightly bound to the cell wall that even purified samples (as determined by SDS-PAGE) contain portions of the cell wall (Lane 1994). Its extracellular location and unusual physiochemical properties have prompted hypotheses suggesting an involvement with cell wall fortification and fungal resistance during germination of the embryo.

In addition to a role in germination, germin-oxalate oxidases are involved in a variety of stress responses. Within the root, germin has been specifically localized to the root surface using activity staining and *in situ* detection of mRNA in root sections (Dumas *et al.* 1995; Caliskan and Cuming 1998). Hurkman and co-workers (Hurkman *et al.* 1991; Hurkman and Tanaka 1996a) showed that salt shock increases both germin polypeptides and mRNA levels in barley roots. Fungal infection was also shown to induce the accumulation of germin polypeptides (Zhang *et al.* 1995; Dumas *et al.* 1995). Using colorimetric assays, Zhang *et al.* (1995) demonstrated that oxalate oxidase activity in barley roots increases 1-3 days before protein accumulation. They later showed that

transcripts encoding the germin-oxalate oxidase also accumulate after fungal invasion (Zhou *et al.* 1998).

More recently, germin was shown to be regulated by heavy metals. Wojtaszek *et al.* (1997) exposed lupin cells to 1 mM $CuCl_2$ and measured changes in exocellular protein composition. They observed an increase in the amount of a 33 kD protein in the growth medium concomitant with a decrease in a 33 kD protein in cell wall fractions. Following amino acid sequencing, both 33 kD polypeptides were identified as germin-oxalate oxidases. No studies were performed to explain this apparent shift in protein location. Berna and Bernier (1999) tested the effects of a variety of heavy metals on the germination of wheat seedlings. Root extracts from seedlings germinated in Cd, Co and Cu (300 μM) contained higher levels of oxalate oxidase activity than those germinated in water. In comparison, Fe, Mn, Ni and Zn treatment during germination had no effect on activity, suggesting that the response is specific to metal treatment. Like the heavy metal response, Al has also been shown to induce oxalate oxidase gene expression in roots. Hamel *et al.* (1998) reported the up-regulation of oxalate oxidase mRNA expression using differential screening of a root tip cDNA library prepared from an Al-tolerant wheat cultivar. The induction of mRNA expression was concluded to be a toxicity response rather than a tolerance mechanism, since cultivars with differential tolerance responded by expressing the same level of transcripts when the same degree of root inhibition was achieved. Using tobacco plants transformed with an oxalate oxidase gene, Nowakowska (1998) further confirmed that Al stress induces both the activity and accumulation of this enzyme in roots that normally do not express activity. In these two

Al studies, assays were performed on proteins extracted from roots grown in Al. No studies have directly assayed enzyme activity in the presence of Al.

4.4. ORIGIN OF EXUDATE PROTEINS

The growth medium bathing the roots of the Al-sensitive wheat (*Triticum aestivum* L.) cultivar Katepwa contained at least 14 polypeptides. These exudate proteins could have four possible origins: 1) microorganisms, 2) seeds, 3) cytoplasmic contamination from damaged or sloughed cells, or 4) roots.

Contamination from microorganisms is an unlikely source of proteins in the growth medium, since all collection steps were performed under as aseptic conditions. To further ensure sterility, aliquots of the medium were plated following each harvest, and only those free of bacterial and fungal growth were used for purification.

Seeds represent a second source of exudate proteins. Seeds are in constant contact with the growth medium and contain a large pool of proteins. After the purified proteins were sequenced, both showed homology to germination-related proteins. This association prompted the analysis of tissue origin. Neither the 24 nor 40 kD proteins were detected in the absence of the seed (Figure 13). Thus, the proteins purified in this work are likely those that leached out of the seed during germination, protein mobilization and degradation. In retrospect, this could explain the continued exudation of the 24 and 40 kD proteins in the presence of Al while most others disappeared concomitant with root stunting.

Cytoplasmic contamination represents a third possible source of protein origin. Proteins could enter the rhizosphere through senescing cells, mechanically damaged cells, or from the sloughing off of root cap cells during active growth (Curl and Truelove 1985). Since the seedlings used in these experiments were relatively young (less than two weeks old), cell turnover in mature regions should have been minimized. Mechanical damage should have also been minimal because experiments were performed in a solution medium rather than soil. Thus, a cytoplasmic origin appears to be unlikely for any of the exudate proteins.

A fourth potential source of exudate protein is the root apoplasm. Roots have been shown to continuously secrete biologically active molecules into their immediate surroundings (see Introduction). Root exudate proteins were identified from the growth medium of seedlings following seed excision. The most prominent root exudate proteins correspond to molecular weights of approximately 22.8, 23.5, 41 and 45 kD (Figure 13). Except for the 22.8 kD band, these proteins were not abundant in the presence of the seed. This can be explained by the greater abundance of seed proteins, which would mask the presence of root exudate proteins. Due to time constraints, no studies could be pursued with these root proteins. Although the 24 kD protein appears to have originated from the seed, I demonstrated that oxalate oxidase activity is also present on the root (Figure 9). This observation has been previously reported (Dumas *et al.* 1995; Caliskan and Cuming 1998). Thus the oxalate oxidase isoforms (seed and root) may represent two potentially important sites of Al injury.

4.5. DIFFICULTIES WITH STUDYING WHEAT EXUDATE PROTEINS

Exudate proteins are inherently difficult to study for several reasons. As mentioned in the Introduction, contamination by microorganisms is a major concern. Since the seeds used in my study were collected from the field, they were laden with bacterial and fungal spores. Contamination could not be eliminated by bleach, detergents (SDS and Triton), ethanol, nor combinations thereof. Ultimately, $HgCl_2$ was the only method that effectively eliminated contamination without hindering seedling growth.

Another difficulty with studying exudate proteins is the large effort required in collecting material. For example, one experiment entailed growing 60 sterile vessels of 5 plants/vessel. Maintenance of such a culture required large amounts of time and careful technique, resulting in a time-consuming and laborious process.

A third difficulty arose from the low quantity of proteins exuded into the rhizosphere. Young seedlings (7 - 14 days old) were used because numerous labs have used seedlings at this stage for analyzing the toxic effects of Al on wheat (e.g. Basu *et al.* 1994a; Pellet *et al.* 1996; Hamel *et al.* 1998). Smaller plants are also easier to maintain under sterile conditions, although difficulty arises because they grow rapidly within a small volume of media. Long-term collection of exudates was not possible since vessels were limited in size (seedlings rapidly outgrew their vessel) and nutrients in the medium were quickly depleted (possibly resulting in the onset of nutrient deficiency symptoms). Continual replenishment of the nutrient medium was difficult since it introduced contamination into the vessel almost regardless of the care taken to avoid it. Media

replenishment also resulted in further dilution of exudate proteins. Consequently, collecting sufficient protein for sequencing required numerous collections. For future studies, difficulties are anticipated for collecting genuine root exudate proteins, since seedlings grow very slowly without the seed and hence will exude even less protein into the media.

The combination of these factors may explain the poor characterization of proteins in the apoplasm/rhizosphere to date.

4.6. ALUMINUM INHIBITION OF OXALATE OXIDASE ACTIVITY

Purification of the seed oxalate oxidase serendipitously spawned my efforts to study the root oxalate oxidase. As mentioned in section 4.3, oxalate oxidase is also found in roots and is involved in many stress responses. Since the root apoplasm has been shown to accumulate up to 99.5 % of cellular Al (Taylor *et al.* 1999) and oxalate oxidase activity has been identified on the root surface (Dumas *et al.* 1995), this enzyme represents a potentially important target for Al injury. An effect of Al on oxalate oxidase during germination and early seedling establishment may also be possible. Recently, oxalate oxidase expression was also shown to be regulated by Al stress (Hamel *et al.* 1998). Thus, the remainder of my research focused on measuring the effect of Al on oxalate oxidase activity.

Histochemical staining confirmed that oxalate oxidase activity is localized in the mature root regions of Katepwa (Al-sensitive) and Maringa (Al-tolerant) seedlings in the absence of Al stress. This activity did not originate from the seed because it was specific

in its localization (Figure 9). If the protein originated from the seed, rinsing in water should have removed the soluble protein. This was not observed. Furthermore, contamination from seed sources would be expected over the full length of the root. Once again, this was not the case. Oxalate oxidase activity staining could not be detected in root tips under non-stress conditions, suggesting that the enzyme is only associated with mature cells. This is in agreement with Caliskan and Cuming (1998), who cited this pattern of activity staining as support for the involvement of germin-oxalate oxidase in restricting cell wall extensibility. In contrast to non-stressed seedlings, roots of the Al-sensitive Katepwa grown in the presence of Al showed staining along the entire root length (tip included). A possible explanation for this observation could be the maturation of cells closer to the root tip, which accompanies the inhibition of cell division by Al. Because mature cells are now located at the root tip, staining becomes evident throughout the whole root length. Other symptoms of Al injury in Katepwa were simultaneously observed, including overall root stunting and stubby lateral root formation. None of these symptoms were evident in Al-treated roots of Maringa. Furthermore, oxalate oxidase activity in Maringa remained localized to mature root regions. This difference in staining patterns in the presence of Al is consistent with Katepwa's classification as a sensitive wheat cultivar relative to Maringa.

I was unable to detect differences in the staining intensity of mature root regions between plants grown in the presence or absence of Al. This may reflect the insensitivity of the histochemical staining technique for detecting degrees of inactivity. Consequently, an *in vitro* assay was used to measure the effect of Al on activity. Oxalate oxidase assays

were performed on a commercial sample of barley oxalate oxidase and the wheat oxalate oxidase present in crude exudates. A large number of published studies have demonstrated similarities between the barley and wheat isoforms, including similarities in protein sequence alignments (Dumas *et al.* 1993), cross-recognition through immunoblotting (Dumas *et al.* 1995), and cross-hybridization with cDNA probes (Hurkman and Tanaka 1996b). Thus, barley oxalate oxidase is a convenient and appropriate analogue for the wheat root oxalate oxidase.

Two active germin-oxalate oxidase bands were detected in the crude exudates (Figure 10). These bands could either correspond to differentially glycosylated G and G' forms reported previously by Lane *et al.* (1986, 1992), or perhaps to different germin-oxalate oxidase isoforms (e.g. seed versus root). The former is more likely for two reasons: 1) removal of the seed eliminates the 24 kD band and oxalate oxidase activity (section 3.8), suggesting that no root exudate isoform is present in detectable quantities, and 2) the root isoform has been shown in the literature to be tightly associated with the cell wall (Lane *et al.* 1992), suggesting that oxalate oxidase is not released from the root. Thus, it is unlikely that oxalate oxidase in the growth media originated in the root. Upon exposure to Al, both the barley and wheat isoforms were inhibited (Figure 11). Although silver staining suggested that less protein was present under conditions of high Al concentration (likely due to protein precipitation), the differences observed were probably too small to account for the differential activity staining. Also, the ratios of activity staining to silver staining decreased with increasing Al. This suggests that oxalate oxidase is a potential target of Al injury.

The *in vivo* effect of Al on oxalate oxidase could be two-fold. First, Al could directly inhibit oxalate oxidase by binding to it. Using a variety of amino acid-specific reagents, Kotsira and Clonis (1997, 1998) demonstrated that sulfhydryl- (possibly disulphide bridges) and carboxyl-groups are crucial to the tertiary structure and activity of oxalate oxidase. Since the chemistry of Al allows for binding to both of these groups, it is possible that Al can bind directly to these sites to inactivate the enzyme. Using sequence information, Gane *et al.* (1998) generated 3D structural models for oxalate oxidase. These putative structures (a homopentamer and a trimer of dimers) both contained a cluster of histidine residues that define a metal-binding location within the oxalate oxidase active site. This site could reflect yet another Al-binding position. Bringezu *et al.* (1999) isolated cell wall material from the metal tolerant *Silene vulgaris* and found it accumulated Fe, Ni, Cu, Al, Sn and Zn. When cell walls were isolated from a *Silene vulgaris* suspension culture and analyzed by copper-activated affinity chromatography, a protein with oxalate oxidase activity and high amino acid sequence homology to germin was isolated. Similarly, my experiments using metal affinity chromatography showed that the 24 kD subunits of oxalate oxidase bind with Al (Figure 4). The bulk of this protein could only be washed from the Al-activated affinity column using a high concentration of EDTA, indicating that oxalate oxidase binds relatively strongly to Al.

Aluminum could also inhibit oxalate oxidase activity by binding to its substrate. Oxalate is an organic anion that binds Al strongly to form stable 5- or 6-bond ring structures (Hue *et al.* 1986). Relative to other organic acids, oxalate is amongst the

strongest detoxifiers of Al (Hue *et al.* 1986). Although oxalate is thought to be involved in the detoxicification of rhizospheric Al in cotton (Hue *et al.* 1986), buckwheat (Zheng *et al.* 1998) and taro (Ma and Miyasaka 1998), it is not a major organic acid in wheat exudates (Basu *et al.* 1994a). Because of its low abundance and relatively strong capacity for binding Al, substrate limitation could also effectively inhibit oxalate oxidase activity *in vivo*. Since no stability constant is known for the oxalate oxidase-Al complex, it is not known whether Al binds stronger to the enzyme or to oxalate. Thus it is difficult to discern which mechanism of inhibition could be responsible for inhibition under environmental conditions.

4.7. SIGNIFICANCE OF OXALATE OXIDASE INHIBITION BY ALUMINUM

A recurrent theme in today's research states that Al *only* affects the actively growing root tip (see Introduction). This concept largely stems from research by Ryan *et al.* (1993), who showed that root growth, cell elongation and cell morphology are not altered unless Al is exposed to the root meristem. The specific markers used in this study for identifying injury, however, may have biased this conclusion. Undifferentiated cells of the meristem are obviously more likely to reflect such injuries (inhibition of mitosis and alteration in morphology) when compared to differentiated cells in mature regions. Perhaps new markers of injury that are relevant to both mature and immature regions of the root need to be identified to truly reflect the occurrence of damage throughout the root. Recent literature has also focussed on finding short-term injuries induced as close to the onset of Al exposure as possible (Rengel 1996). Although the quest for identifying primary Al injuries has merit, such studies cannot provide a complete picture of the Al toxicity syndrome for two reasons: 1) acclimatization to initial injury is possible, and 2)

field performance is dependent on the long-term health and growth of whole root systems (Parker 1995). Despite these observations, little consideration has been given to Al damage in mature root regions. This may also be due in part to the difficulty in distinguishing between the direct and secondary effects of Al. My research on oxalate oxidase presents evidence to support the possible occurrence of lesions in mature tissues. Although assays were performed on enzymes from barley roots and wheat seed exudate, it is predicted the wheat root isoform would respond similarly.

Inhibition of oxalate oxidase by Al could have several repercussions. A failure to generate hydrogen peroxide may be central to most of these injuries. Wheat roots respond to Al by up-regulating oxalate oxidase gene expression (Hamel *et al.* 1998), perhaps in an attempt to compensate for decreased enzyme activity (present study). As already mentioned, hydrogen peroxide provides protection from stress by reinforcing the cell wall via peroxidase-mediated cross-linkage reactions and lignification. If the pool of available hydrogen peroxide decreases, cell wall integrity could be weakened. Inhibition of oxalate oxidase by Al would also render the root more susceptible to fungal infection, since hydrogen peroxide has been cited to induce pathogen resistance (Apostol *et al.* 1989). Another consequence of the Al inhibition of oxalate oxidase is a change in Ca^{2+} pools. Since less oxalate (present as a calcium salt) is degraded, less Ca^{2+} will be released in the apoplasm. This would occur concomitantly with an Al-induced blockage of calcium channels (Rengel 1996). Disruption of Ca^{2+}-mediated homeostasis and the perception of environmental stimuli may follow. Blockage of Ca^{2+} channels by Al probably has a greater effect than the contribution by oxalate oxidase inhibition to Ca^{2+}

homeostasis since wheat does not accumulate large quantities of Ca-oxalate in the apoplasm. Furthermore, the charge gradient, rather than concentration gradient, across the plasma membrane provides the primary driving force for Ca^{2+} influx. Nonetheless, the combination of the H_2O_2 and Ca^{2+} effects associated with oxalate oxidase inhibition could contribute to the root injuries observed in Al soils. Theoretically, Al could also affect these processes within the seed.

4.8. FUTURE WORK

A systematic study of the apoplasm/rhizosphere is necessary before we can fully understand the impact of Al on plants. My analysis of exudate proteins in the growth media identified two sources of origin for these proteins, the seed and the root. Since seed exudate proteins are not likely to contribute to root growth after seedlings are established, Al-binding to root exudate proteins is more likely to cause root-related injuries. For subsequent studies, attention needs to be focused on these root exudate proteins as potential targets of Al injury. This will also require the testing of other methods for collecting and isolating apoplastic proteins that are tightly bound to the cell wall. For example, washing the root surface with a salt solution may release a new pool of rhizosphere proteins. Robertson *et al.* (1997) showed that proteins in the medium of suspension-cultured cells differed greatly from those obtained by a $CaCl_2$ wash. Use of more aggressive reagents or enzymes to degrade the cell wall could also release a new suite of proteins. In relation to the root, each of these pools could contain potentially important proteins inhibited by Al.

In addition to providing insight into Al injury, characterization of the root apoplasm/rhizosphere will yield information on other root processes. To date, relatively little is known about this compartment, and published studies have only identified subclasses of molecules from the apoplasm/rhizosphere of individual plant species. Research of this type provides limited information, since plants exude distinct sets of compounds unique to the species (Gleba *et al.* 1999). This observation further suggests that extrapolation from species to species is of limited value. Another limitation of exudate research done to date is the bias towards identifying molecules of particular interest only to the researcher. Most studies have searched for specific molecules in the rhizosphere, leaving little opportunity for discovering novel compounds that could exist in this unexplored bank of biomolecules. A recent paper on molecular farming even suggests an industry-related significance for root exudation in the future (Gleba *et al.* 1999).

The prediction that Al may inhibit cell wall oxalate oxidase arose serendipitously from my research on exudate proteins. Although my data likely reflects the occurrence of oxalate oxidase from a seed source, the root isoform warrants interest as a possible cell wall lesion. Since no such studies have been published to date, I suggest that experiments similar to those I performed with the exudate isoform (purification and enzyme assays) need to be repeated on the cell wall isoform. For comparison, it would also be interesting to repeat the study using protein isolated from the Al-tolerant cv. Maringa. These formidable tasks will be left to a future researcher.

5. CONCLUDING STATEMENT

My work suggests that exudate proteins can yield new information on the expression of Al injury. Concomitant with a decrease in root elongation and an induction of callose synthesis upon exposure to Al, I observed a dramatic reduction in the number of exudate proteins in the Al-sensitive wheat cultivar Katepwa. Two proteins that remained intact under Al stress and are capable of binding to Al were purified and identified as oxalate oxidase and serpin. Since both proteins appear to have originated from the seed, neither is anticipated to have a long-term effect on root growth, although an effect on germination or early seedling establishment cannot be dismissed. Assays on the exudate oxalate oxidase and barley oxalate oxidase suggest that the root localized-oxalate oxidase may be similarly inhibited. This would represent a new cell wall lesion found in mature tissues. Much work however, needs to be performed for confirmation. Future studies need to directly demonstrate the inhibitory effect of Al on the cell wall isoform. This could help elucidate the impact of Al on oxalate oxidase in roots and seeds. Such studies on the rhizosphere could provide a more complete understanding of Al's toxic effects and also provide new information on this largely understudied compartment.

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